

# Construction of a supercritical fluid extraction (SFE) equipment: validation using annatto and fennel and extract analysis by thin layer chromatography coupled to image

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## Abstract

The present work describes setting up a laboratory unit for supercritical fluid extraction. In addition to its construction, a survey of cost was done to compare the cost of the homemade unit with that of commercial units. The equipment was validated using an extraction of annatto seeds' oil, and the extraction and fractionation of fennel oil were used to validate the two separators; for both systems, the solvent was carbon dioxide. The chemical profiles of annatto and fennel extracts were assessed using thin layer chromatography; the images of the chromatographic plates were processed using the free ImageJ software. The cost survey showed that the homemade equipment has a very low cost (~US\$ 16,000) compared to commercial equipment. The extraction curves of annatto were similar to those obtained in the literature (yield of 3.8% oil). The separators were validated, producing both a 2.5% fraction of fennel seed extract rich in essential oils and another extract fraction composed mainly of oleoresins. The ImageJ software proved to be a low-cost tool for obtaining an initial evaluation of the chemical profile of the extracts.

**Keywords:** supercritical fluid extraction; homemade extraction unit; annatto; fennel; thin layer chromatography; ImageJ software.

**Practical Application:** A low-cost (~US\$ 16,000) homemade supercritical fluid extraction (SFE) unit for the determination of the process parameters in both steps (extraction and separation) was assembled. A list of the major components and the full details of the construction are provided; using them, the SFE unit can be built by any interested researcher. The bixin present in the annatto extract was detected using thin layer chromatography. Use of the ImageJ software made it possible to evaluate the bixin content in the annatto extracts.

## 1 Introduction

Supercritical fluid technology embodies processes that are performed at pressures and temperatures near or above the critical point of a substance. In the early 1970s, the most popular application was supercritical fluid extraction (SFE), which is often referred to as gas extraction (Brunner, 1994) because of the use of carbon dioxide (CO<sub>2</sub>) at pressures and temperatures above the critical point (304.2 K and 73.8 bar). Knowledge of the capacity of some condensable gases to solubilize a variety of chemicals is well documented in the literature. For CO<sub>2</sub>, it has been well documented since the publication of the solubility of several compounds in liquid CO<sub>2</sub> by Francis (1954).

Supercritical fluid technology is viable at an industrial scale for several applications. Nonetheless, to date, the most popular application is SFE from solid matrices. The extraction of bioactives from several plants is an attractive field; currently, in Brazil, conventional methods of extraction are practiced at the industrial level. Nonetheless, there is a richness of research being done in the field of SFE by the Brazilian and Latin American scientific communities (Moraes et al., 2014). However, there are only a few semi-industrial units in Brazil and Latin America; these units have extractors of up to 12 liters, i.e., they are of small

size compared to the industrial units that are in operation in Asia, North America and Europe. However, experimental data should be used to scale up the process. As discussed by Meireles (2003), small-sized extractors (up to 50 mL) can provide data for selecting the process conditions, such as temperature and pressure. A very careful determination of the extract's chemical composition is required to choose the conditions under which the target compound or a mixture of target compounds is obtained. Then, a very preliminary economic feasibility study should be done. To obtain such data, one can use commercial or homemade SFE units. To the best of our knowledge, the first experimental SFE unit in Brazil was assembled by Meireles & Germer (1992). Since then, several other SFE units have been assembled in LASEFI/DEA/FEA/UNICAMP, as reported in the literature (Ferreira et al., 1993; Pasquel et al., 2000; Santos & Meireles, 2013; Zabot et al., 2014).

The installation of homemade equipment for supercritical extraction with CO<sub>2</sub> at a laboratory scale should be a versatile project that is applicable to various raw materials at different extraction conditions, with at least two separators, and should

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allow for adaptations throughout the research to improve functioning (Martínez & Vance, 2008).

Considering that CO<sub>2</sub> reservoirs' (laboratory bottles') pressures are between 50 and 60 bar, supercritical extraction equipment at a laboratory scale should contain a thermostatic bath with a coil in its reservoir to cool the CO<sub>2</sub> and ensure that it is in the liquid phase during pumping (Brunner, 1994; Zetzl et al., 2003). In this way, the auxiliary pumping system for the chilled fluid, which is usually a solution of ethylene glycol, may be used to cool the last separator and help retain more volatile compounds, thereby guaranteeing a higher purity of the CO<sub>2</sub> evaporated at the end of the extraction line and thus facilitating solvent recovery (Reverchon, 1997).

Another component of supercritical extraction equipment that may provide a dual function is the heating bath. It is used to heat the CO<sub>2</sub> after pumping, thus keeping it in the supercritical fluid condition, and is used as an auxiliary system to heat the extractor and first separator jackets, as shown in Figure 1 (Zetzl et al., 2003).

Using this strategy, which makes the heating and cooling components multipurpose, the cost of the thermal exchange components in laboratory units may represent only 20% of the total equipment costs. A limiting factor of this strategy is that because the equipment will have only one source for heating and cooling, the temperatures must be set to the closest values of the CO<sub>2</sub> pumping conditions and vegetable matrix extraction.

For assembling the various components of the supercritical extraction equipment, 30 × 30 × 6000 mm aluminum profiles, angle bars and screws may be used. These structures are light and adjustable to the needs of the projects, have low acquisition costs and represent only 1.7% of the total installation cost.

Considering the need for supercritical extraction equipment to obtain high-quality extracts without a large initial equipment investment, there was a need for a low-cost SFE unit for use in the development of processes. Then, in 1999, a cooperative project between Brazil (CNPq 910016/99-2) and Germany (Ministry of Research and Education BMBF DLR-IB, BRA 098/78) provided the funds to construct four identical low-cost SFE units at approximately US\$ 15,000 each (all taxes included). In Brazil, the following laboratories were involved: LASEFI/DEA/FEA/UNICAMP, LATESC/EQA/CT/UFSC, and LAOS/FEQ/CT/UFPA. In Germany, the laboratory involved was Arbeitsbereich Thermische Verfahrenstechnik (Technical University Hamburg-Harburg (TUHH)). The homemade, low-cost units were used extensively in all four laboratories. After 14 years, the SFE unit of LASEFI was remodeled. The purpose of this paper is to make the construction details widely available to the scientific community and to describe the processes that were used to validate the equipment. To validate the equipment, two raw materials for which experimental data are available in literature were used (Albuquerque & Meireles, 2012; Coelho et al., 2003; Moraes et al., 2015; Moura et al., 2005; Reverchon et al., 1999; Simándi et al., 1999). The two materials have different characteristics: (i) annatto (*Bixa orellana* L.) has the presence of a lipidic fraction that is rich in tocotrienols and a terpenic fraction containing geraniol, and (ii) sweet fennel (*Foeniculum*

vulgare) contains a lipidic fraction and a terpenic fraction, but in this case, formed by a mixture of terpenes (Moura et al., 2005).

## 2 Materials and methods

The SFE-TUHH was designed and built at the "Thermische Verfahrenstechnik" Laboratory of Professor Gerd Brunner at TUHH – Technische Universität Hamburg-Harburg – in 2002. The following parts from this equipment were used to assemble the renovated SFE unit, now denoted as SFE-0.1L: the extractor, the separators and part of the heating and cooling systems (Zetzl et al., 2003).

The SFE-0.1L unit was intended to facilitate the control of the pressures on the separators, to modernize process indicators and to develop a new support structure for the equipment components that was less susceptible to corrosion. The original SFE-TUHH design was maintained.

### 2.1 Equipment setup

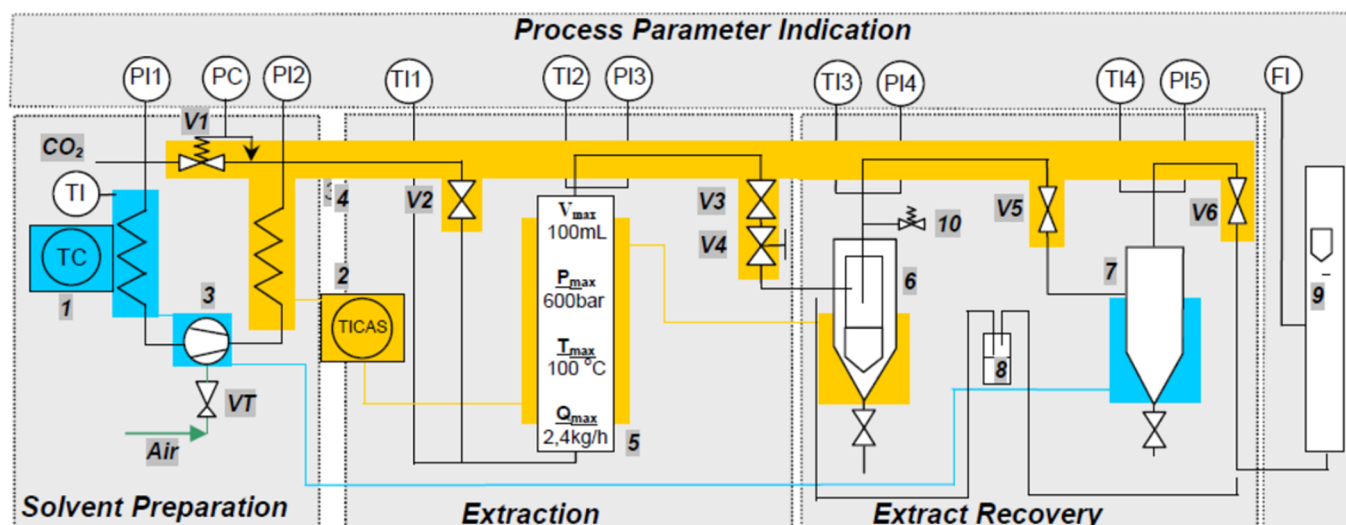
Before starting the renovation of the SFE-TUHH, a list of needed parts was prepared (Table 1). Table 1 lists all of the components used to assemble the SFE-0.1L; the cost of each piece is also presented.

The first step in constructing the SFE-0.1L unit was the assembly of the component support structure using aluminum profiles (30 × 30 × 6000 mm) (SISTEMA, 30 × 30 series, Amparo, Brazil), caps, brackets, screws, bolts and 2 mm stainless steel plates and casters. The main frame of SFE-0.1L contains 4 pieces of 800 mm, 5 pieces of 710 mm and 7 pieces of 510 mm. These pieces units were attached to the primary structure (Figures 2-4). The two steel plates were assembled based on the dimensions of the previously described structure profiles; that is, (770 × 570 × 2 mm) and (300 × 420 × 2 mm). Subsequently, all of the components were located with the objective of easy access to the extractor, separators, valve panel and process indicators. The 1/8-inch stainless steel tubing (Fopil, ASTM A269S, Campinas, Brazil) was folded and reinserted into the structure by connecting the points, in accordance with the flow diagram in Figure 5.

Following the installation of the tubing using the connections and components in Figure 5, thermal insulation was installed. The silicon hoses and their insulation were connected to the heating and cooling components.

The valve box was constructed of stainless steel to avoid corrosion from constant contact with the heating fluid, with 22-mm connections to facilitate fluid output flow. The insulation, similar to all of the heating and cooling components, was made using a 3-mm-thick elastomeric blanket (Epex, Vidoflex M2, Blumenau, Brazil).

In building the unit, a system was installed to reduce pressure variations in the extractor. Pressure variation during the extraction process may cause solute and solvent waste (Martínez & Vance, 2008). Pneumatic pumps may cause pressure oscillations by their own variation in supplying compressed air to the compressor. An efficient system consists of a backpressure valve installed with its inlet after the heating of the CO<sub>2</sub> and the outlet installed before the cooling coil inlet (Zetzl et al., 2003).



1	Cooler (Condenser). CO <sub>2</sub> cooling loop
2	Heater unit
3	Air driven CO <sub>2</sub> Pump ("Booster") 1:110
4	Water-heated box for expansion valves
5	Extraction Autoclave, 100 mL, heating jacket
6	Cyclone with inner parts, heating jacket
7	Separator with cooling jacket
8	Alternative sampling line in glass tubes
9	Flow Meter (kg CO <sub>2</sub> /h) at NPT
10	Safety valve for cyclone and separator
TI 1	Flow Temperature at Autoclave Inlet
TI 2	Flow Temperature at Autoclave Outlet
TI 3	Flow Temperature at Cyclone Outlet
TI 4	Flow Temperature at Separator Outlet

VT	Air Throttle - Booster Piston Frequency
V1	Back Pressure Regulator - Process Pressure
V2	Inlet I/O Valve for Extraction Autoclave
V3	Outlet I/O Valve for Extraction Autoclave
V4	Micro Needle Valve - Fluid Flow Rate
V5	Regulation Valve - Cyclone Pressure
V6	Regulation Valve - Separator Pressure
PI 1	Pressure CO <sub>2</sub> Supply
PI 2	Pressure CO <sub>2</sub> Solvent Preparation Cycle
PI 3	Pressure Extraction Autoclave Outlet
PI 4	Pressure Cyclone
PI 5	Pressure Separator

**Figure 1.** Flow diagram for the Technical University Hamburg Harburg (TUHH) experimental apparatus (Zetzel et al., 2003). The unit assembled in TUHH and send to LASEFI was denoted as SFE-TUHH.

In this way, when the system is over pressure, the pressure valve adjusts, with recycling keeping constant pressure in the extractor. The Maximator M 111 Series pump used to install the SFE-0.1L unit was fixed as close to the thermostatic bath as possible.

Pressurization tests were done on the extraction cells after the equipment was installed. The pressure cells were filled with glass beads and subjected to 350-bar pressure for a static time of 20 minutes, followed by a flow period with solvent flow rate of 15 g/min for 30 minutes. This was done to test the pump's

functioning and to evaluate possible oscillations in the pressure and temperature during the process (Appendix A).

The positioning of the components in the unit prioritized the functionality of the equipment in such a way that all process indicators were visible, easily managed and in front of the operator (Appendix B).

The control and process valves were positioned on the valve panel, with the micrometering valve being responsible for regulating the flow rate (Appendix C). To operate the unit without the separators, auxiliary tubing was developed that connected

**Table 1.** SFE-0.1L Estimated Cost and Components.

Product Name	Qty	Price	Total (USD)
Extractor 100 mL*	1	748.40	748.40
Separator 90 mL*	2	916.80	1,833.61
Jacket*	3	100.03	300.09
Valve box (Maq'nagua, Serra Negra, Brazil)	1	157.23	157.23
Aluminum plate (m <sup>2</sup> ) (Autic, Campinas, Brazil)	3	30.50	91.51
Aluminum profile 6 m (Sistema, 30 × 30 series, Amparo, Brazil)	8.5	11.32	96.23
Angle (Sistema, 30 × 30 series, Amparo, Brazil)	40	1.57	62.89
Square nut and screws (Sistema, 30 × 30 series, Amparo, Brazil)	150	0.68	101.42
End cap (Sistema, 30 × 30 series, Amparo, Brazil)	8	0.79	6.29
Swivel caster (Colson, 3", Araucária, Brazil)	4	6.92	27.67
Manometer* (WIKA, EN8371-1, Klingenberg, Alemanha)	5	341.19	1,705.97
Safety valve (Swagelok, SS-4R3AS, São Paulo, Brazil)	1	237.23	237.23
Temperature indicator (Pyrotec, T4WM-TP100, Campinas, Brazil)	1	190.25	190.25
Termocouple (Pyrotec, TP100, Campinas, Brazil)	5	20.35	101.73
Flowmeter (Cole Parmer, PMR1, IL, USA)	1	336.48	336.48
Flow totalizer (Itrón, G25, Americana, Brazil)	1	40.88	40.88
Tubing 1/8" (6 m) (Fopil, ASTM A269S, Campinas, Brazil)	2	89.62	179.25
Ferrule 1/8" (Fopil, Campinas, Brazil)	10	3.24	32.42
Connector Tee OD 1/8" (Fopil, ASTM A276, Campinas, Brazil)	8	44.30	354.39
Connector Tee side NPT 1/4" (Fopil, A.276316, Campinas, Brazil)	4	20.13	80.50
Connector OD-OD (Fopil, ASTM A269TP316S, Campinas, Brazil)	3	20.12	60.35
Connector OD-NPT (Fopil, ASTM A269TP316S, Campinas, Brazil)	2	11.80	23.60
CO <sub>2</sub> filter (Swagelok, F series, São Paulo, Brazil)	1	96.38	96.38
Blocking valve (Autoclave Engineers, 10V2071, PA, USA)	5	165.17	825.83
Micrometering valve (Autoclave Engineers, 10VRM2812, PA, USA)	1	466.53	466.53
Back pressure valve (Tescon, 44-1800, Sorocaba, Brazil)	1	366.04	366.04
Back pressure valve (Tescon, 44-2200, Sorocaba, Brazil)	1	602.91	602.91
Back pressure valve (Tescon, 26-1700, Sorocaba, Brazil)	1	1,550.33	1,550.33
Air pressure regulator (Norgren, R07-100-RNKA, São Paulo, Brazil)	1	100.94	100.94
Air-driven CO <sub>2</sub> pump (Maximator, M-111L, Nordhausen, Germany)	1	1,861.64	1,861.64
Heating bath (Thermo Haake, DC30/DL30, Eindhoven, Holanda)	1	1,532.84	1,532.84
Cooling bath (Thermo Haake, C10, Eindhoven, Holanda)	1	1,691.82	1,691.82
Ball valve (Japi, ball, Jundiá, Brazil)	2	7.36	14.72
Hose npt adapter (Amanco, Campinas, Brazil)	2	3.46	6.92
Hose connector 90 elbow (Elgo, 1203, Itu, Brazil)	1	1.73	1.73
Insulation tube Ø - 1/8" (m) (Armaflex, AFM-10, Campinas, Brazil)	1	1.83	1.83
Insulation sheets (Epex, Vidoflex M2, Blumenau, Brazil)	0.6	24.45	14.67
Insulation tube Ø - 22 mm (Isolan, Campinas, Brazil)	5	0.94	4.72
Plastic tee conector (Amanco, Campinas, Brazil)	4	1.96	7.83
Silicone hose (5 m) (Sinergia, 200 × 200, Campinas, Brazil)	1	22.17	22.17
		Total	15,938.24

\*Calculated Cost.

the output of the micrometering valve with the external part of the valve panel, thus allowing the extracts to be collected immediately after extraction (Appendix D).

## 2.2 Equipment validation

To validate the equipment, two raw materials, annatto and sweet fennel, were used. The annatto was used to validate the extraction line without the use of the separators, and the fennel was used to validate the extraction followed by separation.

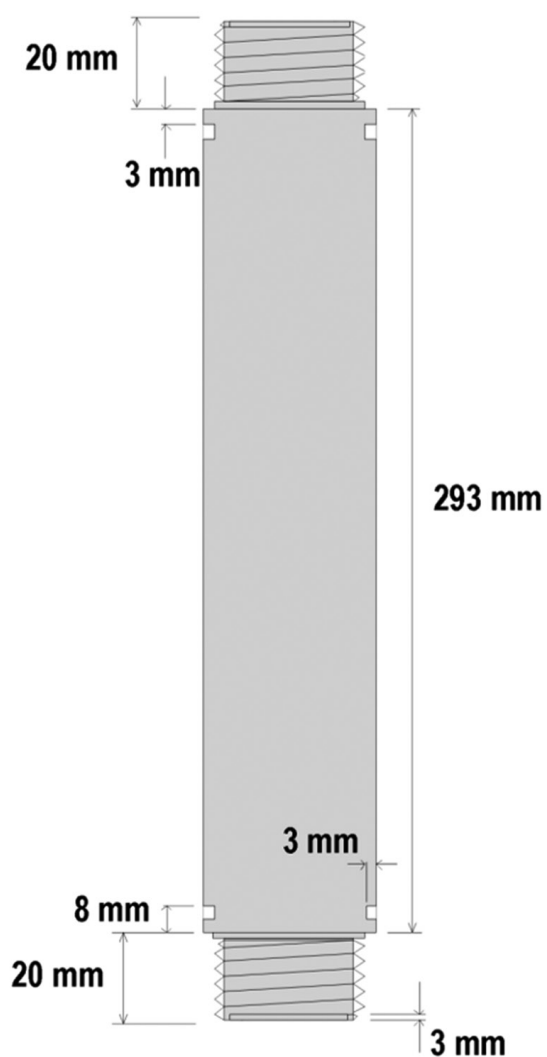
## 2.3 Raw materials preparation

The annatto seeds were acquired at the Estação do Grão Ltda. (São Paulo, Brazil) and stored at 255 K. They were packed into the extractor without any further preparation, as indicated by Silva et al. (2008), Albuquerque & Meireles (2012) and Moraes et al. (2015).

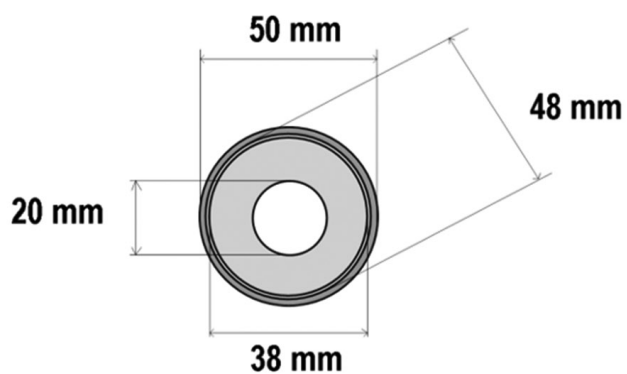
The fennel seeds were acquired in the municipal market of Campinas, Brazil at "Temperos Brasil" and kept at 255 K. The fennel seed preparation procedure was adapted from the



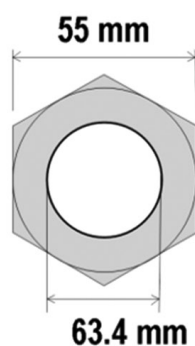
**Extractor - front view:**



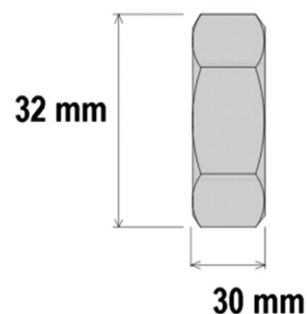
**Extractor - top view:**



**Hex Nut - top view:**



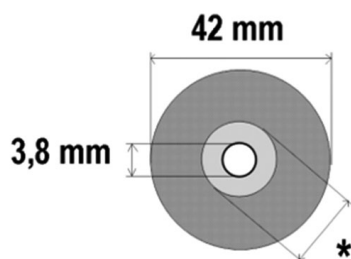
**Hex Nut - front view:**



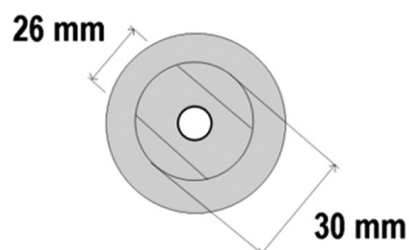
**Nut cover - front view:**



**Nut cover - bottom view:**



**Nut cover - top view:**



● → Circular groove

\* Extractor Measure → 20 mm

\* Separator Measure → 25 mm

**Figure 2.** SFE-0.1L extractor and nuts.

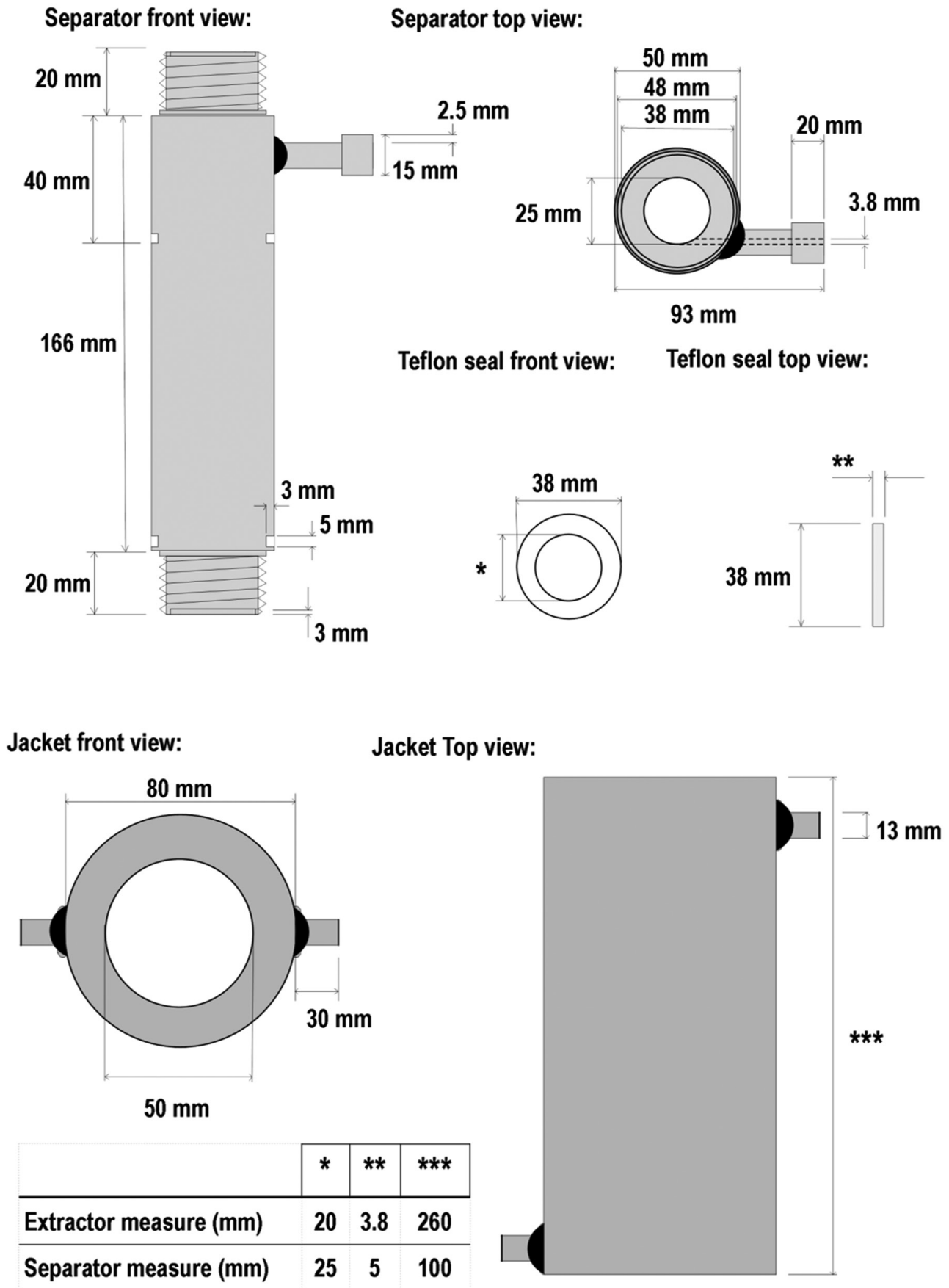
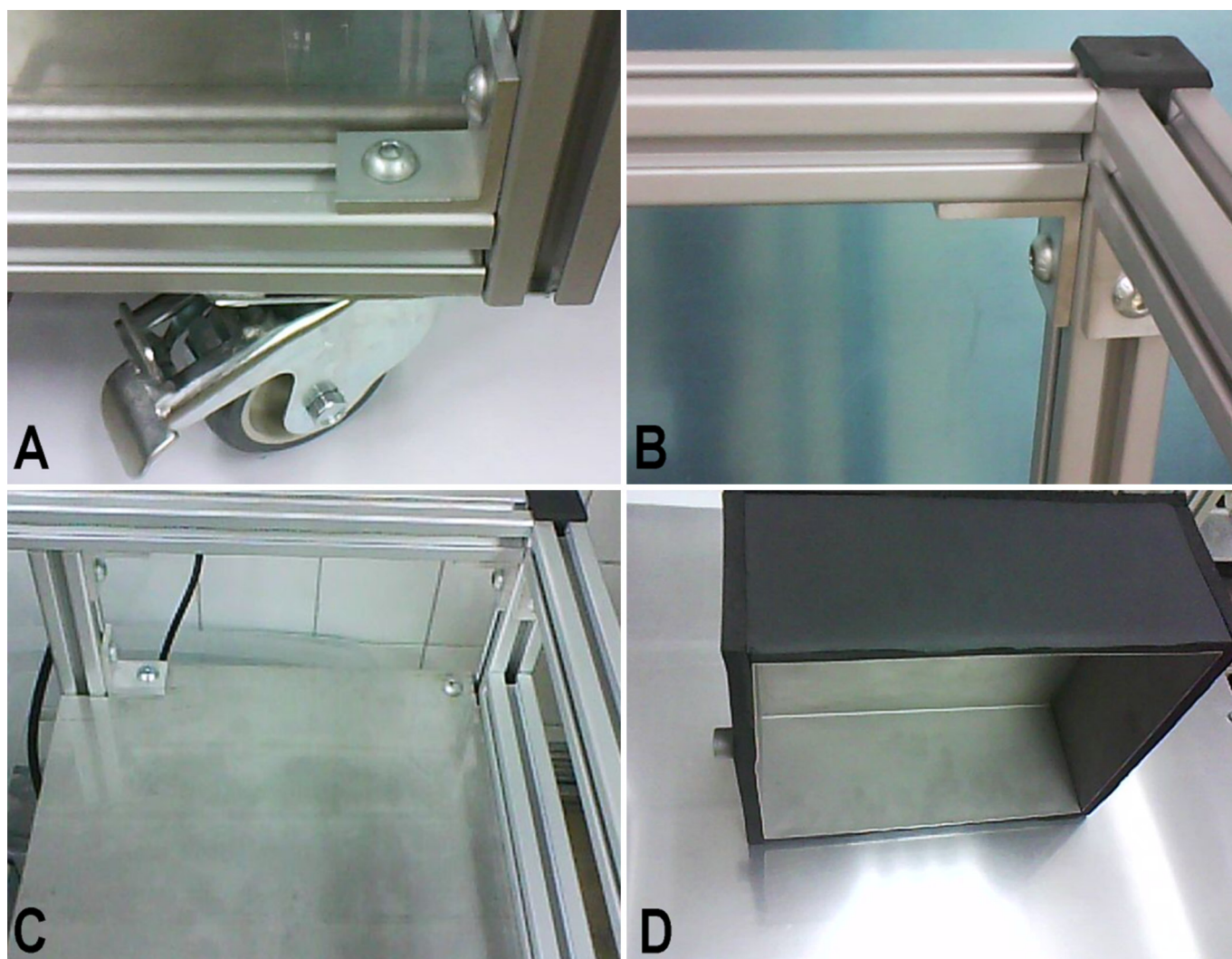


Figure 3. SFE-0.1L separators, teflon seal and jacket.



**Figure 4.** Photos of the structure: (A) caster; (B) aluminum profile; (C) valve box base; (D) valve box.

work of Moura et al. (2005), with the frozen seeds being ground in a mill (Marconi, model 340, São Paulo, Brazil) using a 3-mm sieve into 20-g batches. To characterize the average particulate diameter by the standard method of ASABE (American Society of Agricultural and Biological Engineers, 2008), an electromagnetic agitator was used (Bertel, model 602, São Paulo, Brazil) with screens of the Tyler mesh series (16, 24, 32, 48 and 80 mesh). The prepared seeds were then packed into the extractor.

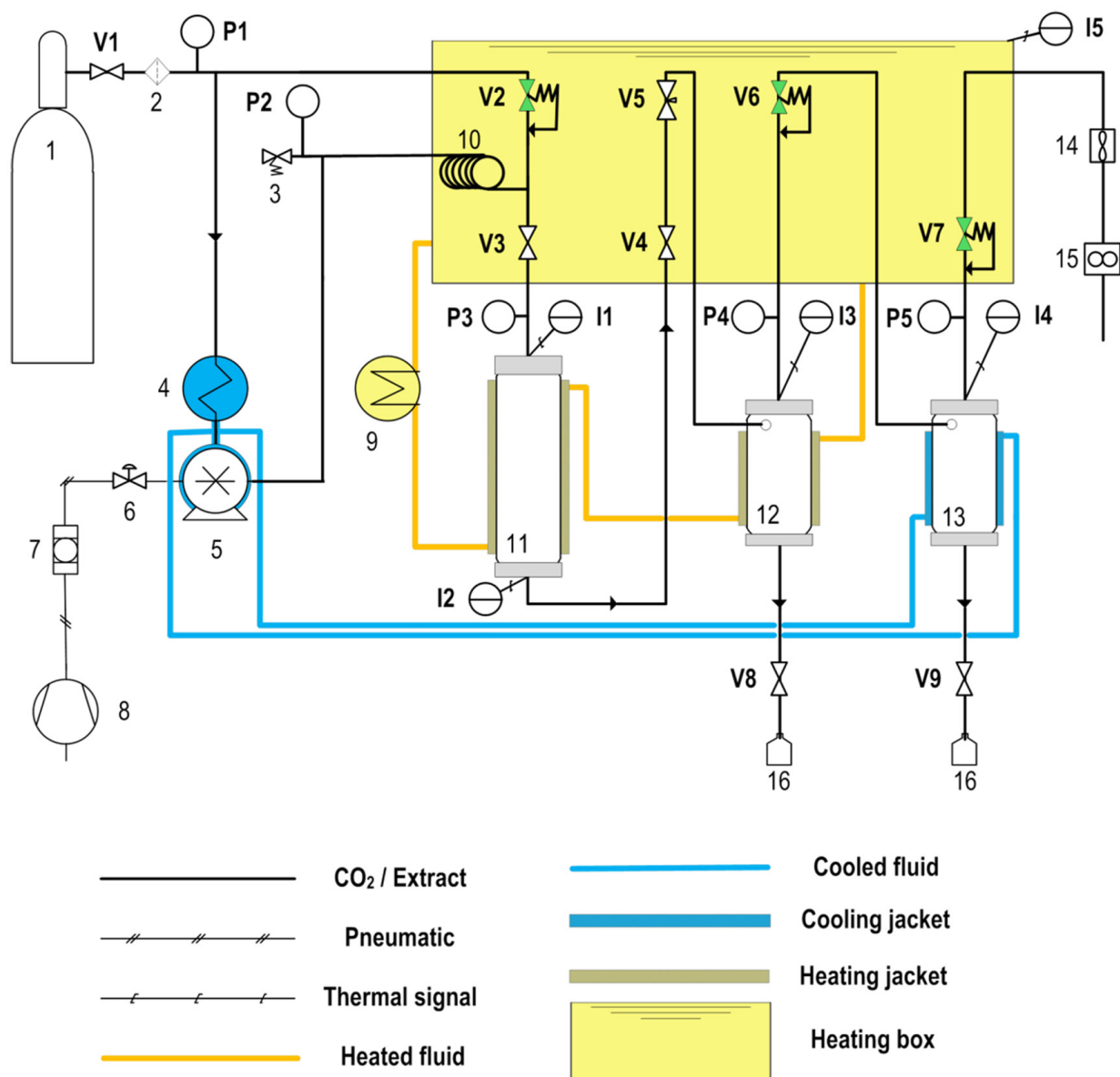
#### 2.4 Extraction and separation

The extractor of the SFE-0.1L unit has a volume of 100 mL and an internal height/diameter relationship of 19 ( $H_B/D_B = 19$ ). The two separators have a 90 mL volume, with a tangential entrance equipped with thermocouples that reach half the height of the cylinder, allowing estimates of the wall boundary conditions and of the temperatures at the central points of the cylinders.

For annatto and fennel, the solvent used was carbon dioxide (99% purity, White Martins, Brazil); the bath was set at 268 K to assure  $\text{CO}_2$  in the liquid phase at the pump entrance. The extractor

pressure was 200 bar, and the temperature was 313 K according to the procedure used by Moraes et al. (2015), with a flow rate of 23.7 g/min and 60 g feeding into the extractor. The extracts were collected immediately after the micrometering valve using glass flasks that were also connected to the flowmeter (Cole Parmer, PMR1, IL, USA) and then to the flow totalizer (Itrón, G25, Americana, Brazil). The ratio of the mass of solvent to the feed mass (S/F) was comparable to the extraction curve of the SFE-0.1L unit for the annatto extraction data of the SFE-2×1L unit, built and validated at LASEFI/DEA/FEA/UNICAMP (Zabot et al., 2014).

The extract masses were weighed, and the extraction yield was calculated as the mass of extract divided by the mass of feed (dry basis). The experimental overall extraction curve (OEC), that is, the cumulated mass of extract versus the extraction time, can be interpreted as containing three stages: (i) the constant extraction rate period (CER), where convection is the major mass transfer mechanism; (ii) the falling extraction rate period (FER), which is dominated by convection and diffusion; and the last stage, (iii) the diffusion-controlled period (DC), where the



List of equipments				List of valves	
1	CO <sub>2</sub> reservoir	11	Extractor	V(1,3,4)	Blocking valve
2	CO <sub>2</sub> filter	12,13	Separator	V(8,9)	Blocking valve
4	Cooling bath	16	Extrac collecting vessel	V2	Back pressure (600 bar)
5	Air-driven CO <sub>2</sub> pump	List of instruments		V6	Back pressure (400 bar)
7	Air filter			V7	Back pressure (100 bar)
8	Air compressor	14	Flowmeter	V5	Micrometering valve
9	Heating bath	15	Flow totalizer	3	Safety valve
		I(1,2,3,4,5) - Temperature Indicator		6	Control (air flow)
10	Serpentine tube	P(1,2,3,4,5) - Pressure gauge			

Figure 5. Flow diagram for the SFE-0.1L experimental apparatus.



diffusion of solute from the solid structures defines the mass transfer rate (Meireles, 2008). The overall extraction curve (OEC) was adjusted to a spline model containing three straight lines using the SAS 9.2® software according to the steps described by Meireles (2008) and Zabot et al. (2014).

Fennel extract was obtained at 200 bar and 313 K according to the procedure used by Reverchon et al. (1999), with a flow rate of 12 g/min and S/F of 10. The conditions in separators 1 (80 bar, 312 K) and 2 (20 bar, 279 K) were adapted from the works of Coelho et al. (2003) and Simándi et al. (1999). After 60 minutes of extraction, the extracts were stored at 255 K for later analyses.

## 2.5 Analyses of extracts

**Annatto extracts:** Extracts were analyzed using Thin Layer Chromatography (TLC). A bixin standard was prepared according to the procedure performed by Albuquerque & Meireles (2012), using a Shaker Incubator (Marconi, MA420, São Paulo, Brazil) in which annatto seeds were exhaustively extracted with acetone for 6 h (200 rpm, 298 K). TLC plates (Macherey-Nagel DC-Fertigfolien Alugram®, XTSILG, Darmstadt, Germany) were used; the mobile phase was hexane:ethyl acetate:formic acid (79:18:3), as optimized by Albuquerque (2013). For fennel extracts, the TLC plates (Macherey-Nagel DC-Fertigfolien Alugram®, XTSILG/UV<sub>254</sub>, Darmstadt, Germany) were used; the mobile phase was toluene:ethyl acetate (6:4), as described by Wagner & Bladt (2001).

The pattern of bixin (3 µL) was applied 3 times on the chromatographic plate to test the repeatability of the method, and 6 points of this pattern with different masses of bixin (0.6, 1.2, 2.4, 4.8, 9.6 and 19.2 µg) were applied on the same plate to evaluate the linearity of the developed method.

The pattern of bixin (3 µL) was inserted at the first point of the plate, followed by the extracts of annatto that were collected at selected extraction times. The extracts (1 µL) were inserted directly onto the plates based on the increasing sequence of extraction times. The plates were photographed with and without the developer AS - anisaldehyde sulfuric acid reagent - and heated for 5 min at 383 K using a tank equipped with a UV lamp: Short Wave (254 nm) - Long Wave (366 nm) - (Entela, UVGL-58, USA).

The fennel extracts obtained from the first and second separator were diluted with toluene in the proportion extract:toluene (1:1); 1 µL of the extract was used (extract in separator-1) with 3 repeated applications of 1 µL for the extract from separator-2. The eluted plates were photographed in a tank equipped with a UV light (254 and 366 nm). The plates were heated for 5 min at 383 K before and after spraying with concentrated sulfuric acid developer.

The images of the chromatographic plates were processed using the free ImageJ software (Ferreira & Rasband, 2010). A location with adequate illumination for the possible points was prepared first, so the areas of the plate with less illumination would not influence the images. A standard photodocumentation height, the point where the plate was placed and the point where

the machine would be positioned to take photos, was selected. The camera was fixed using a support consisting of a stand with handles, with the lens and the TLC plate remaining parallel and at a distance of 30 cm. Subsequently, the photos were opened in ImageJ by the command <File-Open>. A plate without any extract was tested to evaluate the efficiency of the photodocumentation system, aiming for an image with a standard open surface plane using the Interactive 3D Surface Plot tool.

ImageJ was used to calculate the “Ratio to Front” ( $R_f$ ) of the plates, first testing to find the best way to visualize the bands on the plate. By selecting the command <Edit-Invert> to invert the colors of the plate and comparing the result with the original colors of the image, this tool may provide a more accurate measurement, even for compounds that are present at low concentrations or for those with coloration bands close to the intensity of the pixels on the bottom of the plate.

The best image for measuring the  $R_f$  of each compound was determined by the commands <\*Straight\*, segmented of freehand lines - Analyze - Measure (Results) - Analyze - Label>, which were repeated until all necessary measurements were obtained. During the measurement, the lines were traced without deviating from the slope of the straight line; that information was provided along with the results of the measurements.

The areas of the peaks were calculated to estimate the effect of different bixin concentrations on the method validation curve and to evaluate the bixin content present in each sample of the annatto extracts. The images had a rectangular section of the same  $R_f$  selected, and the areas in the pixels of the compounds were calculated using the tools <\*Rectangular\* - Analyze - Gels - Select First Lane - Analyze - Gels - Plot lanes (Plots) - Wand (Tracing) tool (Results, Area)>.

The 3D surfaces of the bands of the different compounds were plotted using the tools <\*Rectangular\* - Analyze - Gels - Select first lane - Plugins - 3D - Interactive 3D Surface Plot - Select: Filled, (Original Colors, Spectrum LUT or Thermal LUT) and adjusted Grid Size, Smoothing, Perspective, Lighting, Invert, Scale, Z-Scale, Max and Min>.

## 3 Results and discussion

The equipment represented an initial cost of US\$ 15,938.24 with the separators and US\$ 11,979.84 with the extractor only, using the secondary extraction line (Table 2). The extraction and separation cells, valves and thermostatic baths constituted 60% of the total setup cost of the equipment.

The comparison between the costs of acquisition and setup of the laboratory equipment for extraction with supercritical fluid is the principal result of this work, demonstrating that the design of the SFE unit resulted in low-cost equipment.

Commercial unit I had a higher acquisition cost (Table 2) than the other equipment, although some characteristics of the equipment make a direct comparison impossible. The extractor of commercial equipment I has a volume 5 times larger than that of the other units and an electric pumping system that eliminates the need for an air compressor; additionally, the unit is automated and can use a cosolvent in the extraction process.

Non-automated units have lower acquisition costs relative to automated equipment. For example, Commercial unit II may be automated for an additional US\$ 10,850.00 (Table 2).

Comparing the commercial unit of lowest cost, Commercial unit III, to the SFE-0.1L unit without the separators (for both to have the same functionality), the cost of the homemade equipment was 36% of the cost of the commercial unit. Another advantage of the assembly process compared to purchasing commercial equipment is that each project may be adjusted in accordance with the peculiarities of the research objective that will be addressed when using the equipment.

### 3.1 Equipment setup

Figures 6 and 7 show the SFE-0.1L unit and the component positioning in the aluminum structure. Casters were placed on the lower part of the aluminum structure to facilitate moving the equipment for potential maintenance.

The assembly of the two component support plates could have been achieved with 5-mm-thick aluminum plates to facilitate plate perforation to place their fixing screws. The aluminum plates are light, cost less and easy to handle; moreover, they can support the weight of the two baths in the lower part of the unit and the valve box in the upper part of the unit. Though other materials may be used to support the structural components, stainless steel plates were used in this project because of the availability of this material in our laboratory.

In the lower part of the separators, it is possible to observe an adjustable support for extract collection that occurs sequentially in the V7 and V8 shut-off valves. In this way, it is possible to adjust the height of the support in accordance with the type of bottle that will be used in the extract collection process and to release the extract output by opening only valves V7 and V8.

The extraction and separation cells were aligned with the most external part of the structure and were positioned with the indentation at the front of the structure. This helped reduce

the final size of the equipment and the length of the pipes. The equipment measures  $57 \times 77 \times 115$  cm.

Another important aspect of the SFE-0.1L unit is that all of the system valves, including the additional valves, such as a backpressure valve for each separator, make it possible to control the cascade of pressures in the separators; the micrometering valve allows control of the solvent flow rate. The use of a backpressure valve for each pressure cell allows precise control of the working pressures. The system that heats the valves using a fluid-flow heat exchanger proved more efficient than the use of electric resistances. The unit has a safety valve that opens at pressures higher than 450 bar, positioned as shown in Figure 5, immediately after the pump and with the relief directed to the rear of the equipment.

### 3.2 Equipment validation

#### *Extractor validation using annatto seeds*

The extraction yield of the annatto in the SFE-0.1L unit was 3.8%, comparable to the work of Albuquerque & Meireles (2012), who determined a yield of 3.7%. After 15 minutes of extraction, 74% of all annatto extract was collected; nonetheless, the extraction continued for 80 minutes to complete depletion of the raw material (Figure 8-I). The elevated flow rate (12 g/min) used to validate the equipment resulted in an S/F of 37 at the end of extraction.

Figure 8-II shows the experimental values for extraction performed in the SFE-2×1L (Moraes et al., 2013) and in the SFE-0.1L, plotted as a function of S/F. Though the extractors were 10 times farther apart, the results validated the SFE-0.1 unit because of the overlap of the OECs.

The annatto OEC (Figure 8-I and 8-II) can be fitted to a spline with 3 straight lines ((Meireles, 2008), as shown in Figure 8; the adjusted parameters are given in Table 3. With these results, the SFE-0.1L unit can be considered validated. The SFE-0.1L unit can be expanded to a unit with an extractor of up to 1 liter by adjusting only the supports and the thermal shielding.

**Table 2.** Comparison of the amounts spent assembling the SFE-0.1L unit relative to the cost of acquiring commercial units.

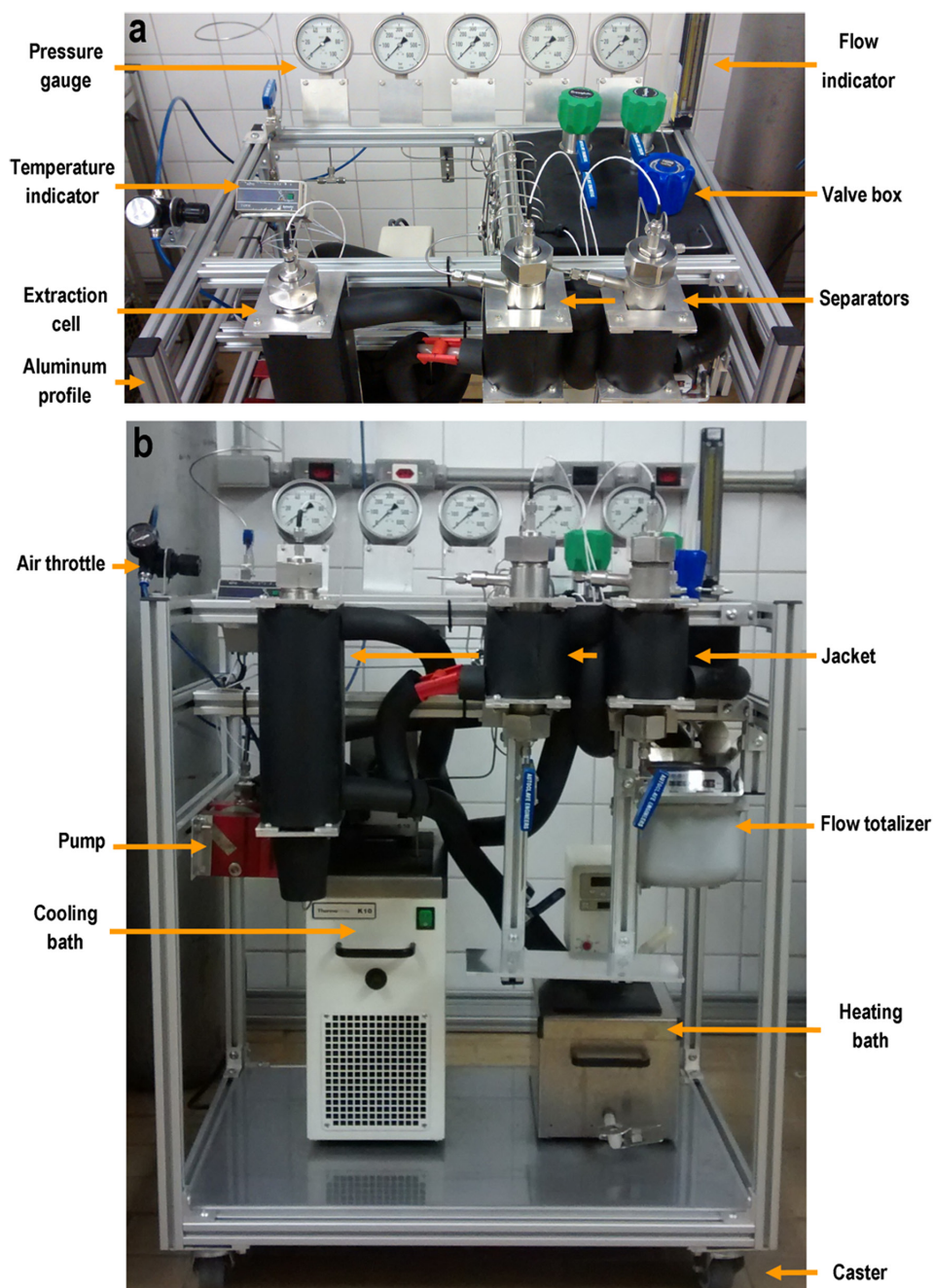
Equipment	Commercial I	Commercial II	Commercial III	SFE-0.1L*	SFE-0.1L**
Extractor Volume	500 mL	100 mL	100 mL	100 mL	100 mL
Number of Separators	2	2	Not applicable	Not applicable	2
Rate of flow	200 g/min	100 g/min	0.01-27 g/min	1.8-30 g/min	1.8-30 g/min
Applicable extractors	500-1000 mL	10-500 mL	5-100 mL	10-1000 mL	10-1000 mL
Cosolvent	Inclusion	Applicable	Applicable	Applicable	Applicable
Operation	Automated	Applicable	Manual	Manual	Manual
Price (USD)	\$121,991.50	\$48,717.00	\$33,145.36	\$11,979.84	\$15,938.24

\*SFE-0.1L unit assembled without separators. \*\*SFE-0.1L unit assembled with two separators.

**Table 3.** Parameters from Annatto Extraction Curve.

	$b_0$	$b_1$	$b_2$	$b_3$	$c_1$	$c_2$	SS
Values	0.015	0.4868	0.0597	0.014	4.4435	18.046	0.0203

$b_0$  – Linear coefficient from straight line 1 (CER);  $b_1$  – Angular coefficient from straight line 1 (CER);  $b_2$  – Angular coefficient from straight line 2 (FER);  $b_3$  – Angular coefficient from straight line 3 (DC);  $C_1$  – Intercept of the first and second straight lines ( $t_{\text{CER}}$ );  $C_2$  – Intercept of the second and third straight lines ( $t_{\text{FER}}$ ); SS – Sum of Squares.



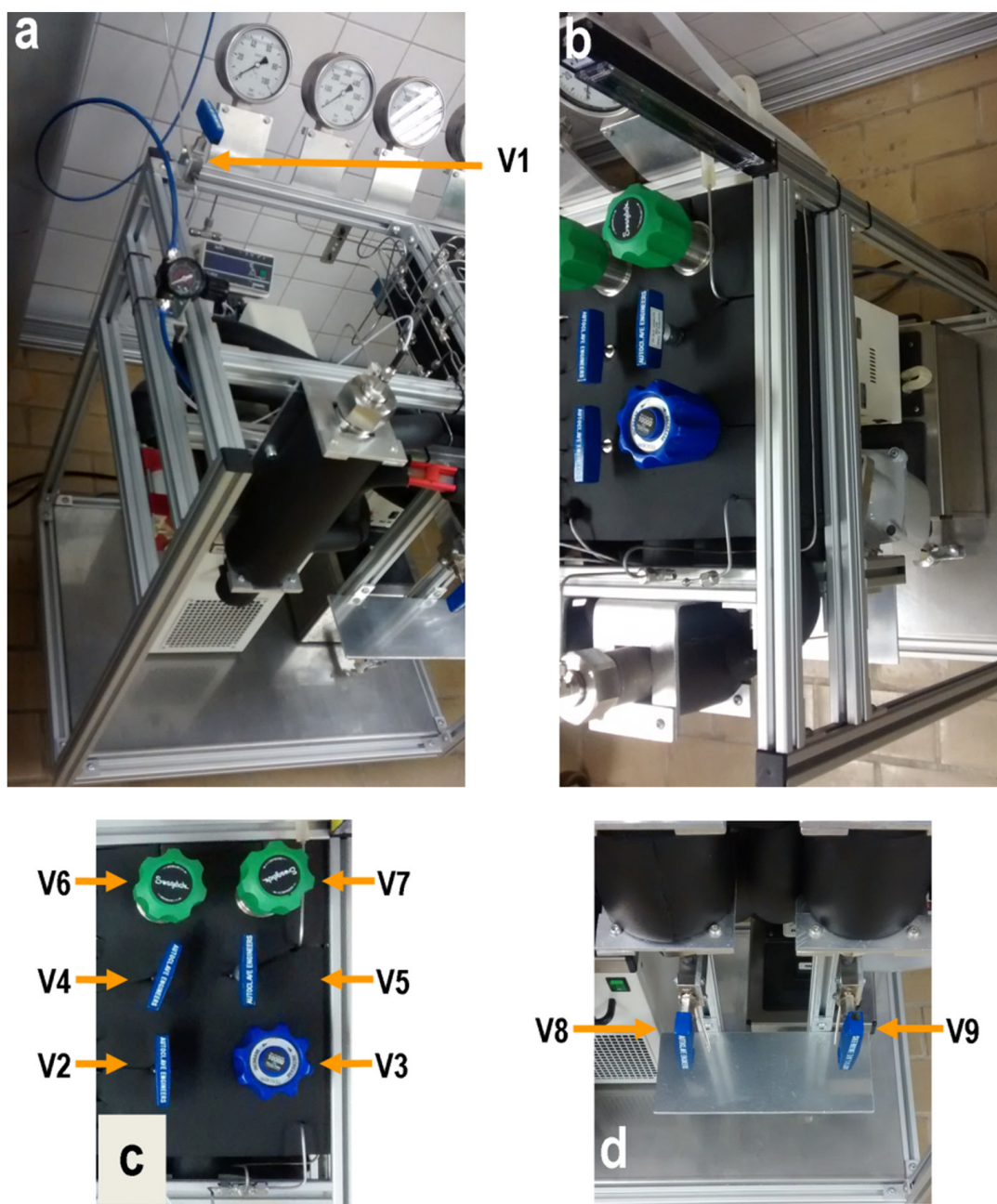
**Figure 6.** Photos of the SFE-0.1L unit, a – top view and b – front view, validating the equipment.

#### *Validation of the separators using fennel*

The commercial equipment from Thar Technologies (model SFE-2×5LF-2-FMC, Pittsburgh, PA, USA) has 2 extractors of 5 liters and 3 separators with volumes of approximately 500 mL. Considering the possibility of using a larger capacity extractor in the SFE-0.1L unit, the separators were designed with volumes of 90% of that of the extractors. The reasoning behind this choice was to ensure that the separators would hold as much extract, in terms of capacity, as would be obtained from a raw material with up to 30% of solute. Considering that, on average, each 100 mL extraction cell may contain approximately 70 grams of

raw material for a 3.8% yield, approximately 2.7 grams of extract would be obtained. Otherwise, for a raw material having 30% of soluble material using a 1-liter extractor capable of processing 700 g of raw material, the extract mass would be ~ 270 grams. Therefore, in this case, the separators must be capable of holding this amount of extract, and separators of approximately 100 mL would be required. This estimate takes into account the fact that the extract is continuously collected. Therefore, for the SFE0.1L unit, 90 mL separators were used, representing 90% of the capacity of the extractor; the larger capacity of the separators thus leaves open the possibility of using a larger extractor.





a		c	
V1	CO <sub>2</sub> Blocking Valve	V2,V4	Extractor Inlet and Outlet Blocking Valves
d		V5	Micrometering Valve
V8	Separator 1 Outlet Blocking	V3	Back Pressure Regulator for Extraction
V9	Separator 2 Outlet Blocking	V6,V7	Back Pressure Regulator for Separation

**Figure 7.** Photos of the SFE-0.1L valves, (a) left side view; (b) right side view; (c) top view and (d) front view.



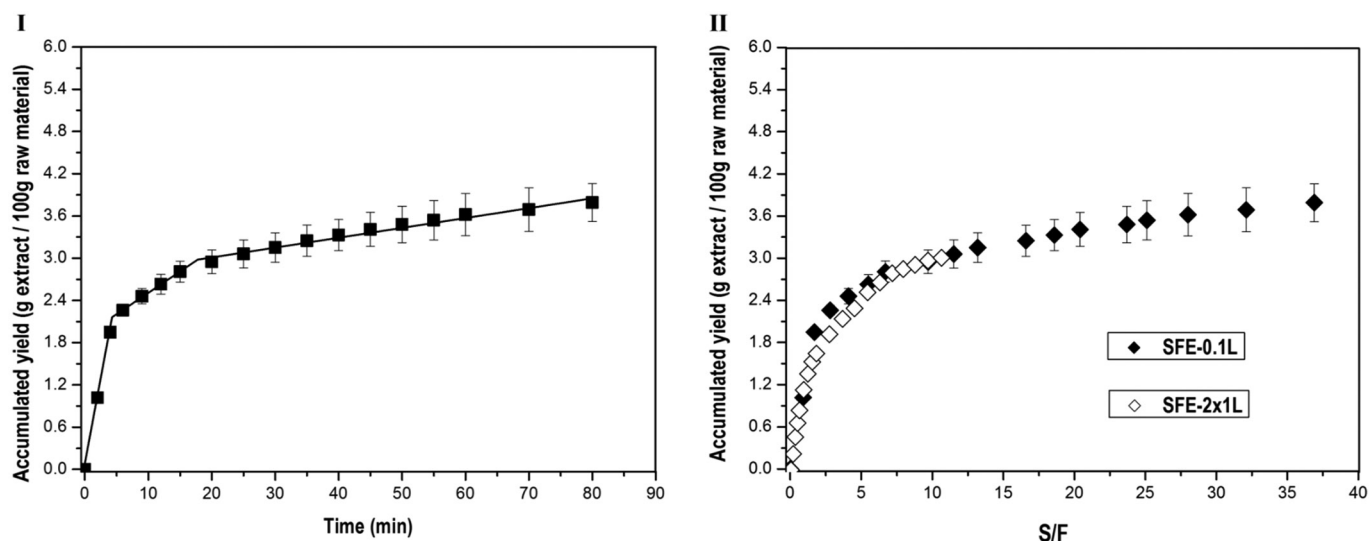


Figure 8. Overall extraction curve obtained on SFE-0.1L equipment and kinetic curves of validating the equipment.

Another important aspect to be considered in the setup of the equipment is that many of the raw materials have a group of majoritarian components. In other words, the division of the extract in the separators may only be 3% for one of the groups of compounds; therefore, the other 97% should be retained in the other separator. The work of Coşge et al. (2008) evaluated the composition of the sweet fennel seed oil and found that sweet fennel has a relationship of 93:3 of oleoresin:essential oil.

There are little data in the literature on the separator volume compared to the extractor's capacity, and the actual construction of equipment at laboratory scale can now serve as an initial parameter for pilot-scale applications.

The fennel extraction in the SFE0.1L unit resulted in a yield of 2.8% (g extract/100 g of ground seeds). As discussed in the next section, this extract mass contained 97.5% of a lipidic fraction collected in the first separator; the remaining 2.5% was the volatile oil collected in the second separator. Stefanini et al. (2006) determined yields of 1.54 to 2.2% using hydrodistillation to obtain fennel volatile oil; both the yield and composition varied with the crop conditions. The higher yield obtained in this work is because CO<sub>2</sub> extracts the lipidic fraction in addition to the volatile oil.

### 3.3 Analyses of extracts

The method used to determine the bixin concentration showed linearity for concentrations between 0.6 to 9.6 µg, as shown in Figure 9E, and had a repeatability that varied by 3.4%, as observed by comparing the areas of the pixels of Figures 9A and 9B.

In Figure 10, the presence of bixin is observed in all of the extracts collected during the extraction; nonetheless, it is important to acknowledge that annatto extract contains other carotenoids that are present in the seeds (Mercadante & Pfander, 2001). Although TLC is a technique that is used mainly in qualitative analysis, it is possible to observe a tendency for the

bixin concentration to increase throughout extract collection at an  $R_f$  of 0.1, as shown in Figure 10A and plotted in Figure 11. In a similar manner, Albuquerque et al. (2015) found an increase in bixin concentration as a function of extraction time.

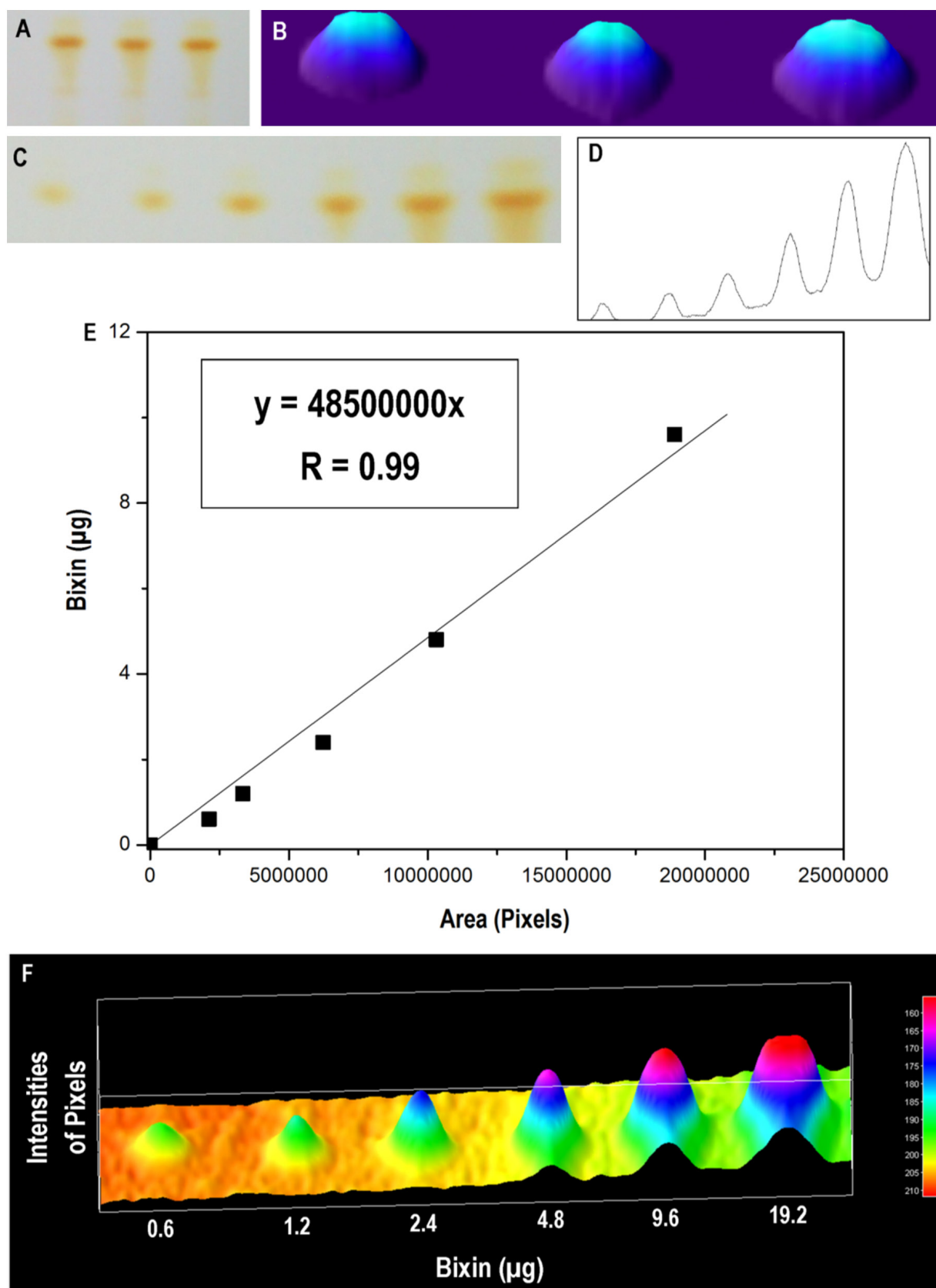
Validating the bixin quantification method by TLC using the ImageJ software made it possible to estimate the carotenoid concentration as a function of the extraction time. In this way, in addition to polling by separation of the present compounds, it was possible to estimate the bixin extraction curve.

Albuquerque & Meireles (2012) obtained annatto extract using a commercial SFE unit Spe-ed (Laboratory System 7071, Applied Separation, Allentown, USA) with a 0.29 L extractor. The data obtained from the extraction on the SFE-0.1L unit with the same raw material is an additional important aspect in validating the equipment: not only was a similar OEC determined, but the extract composition during the extraction was also similar to that obtained in the commercial unit (Figure 8).

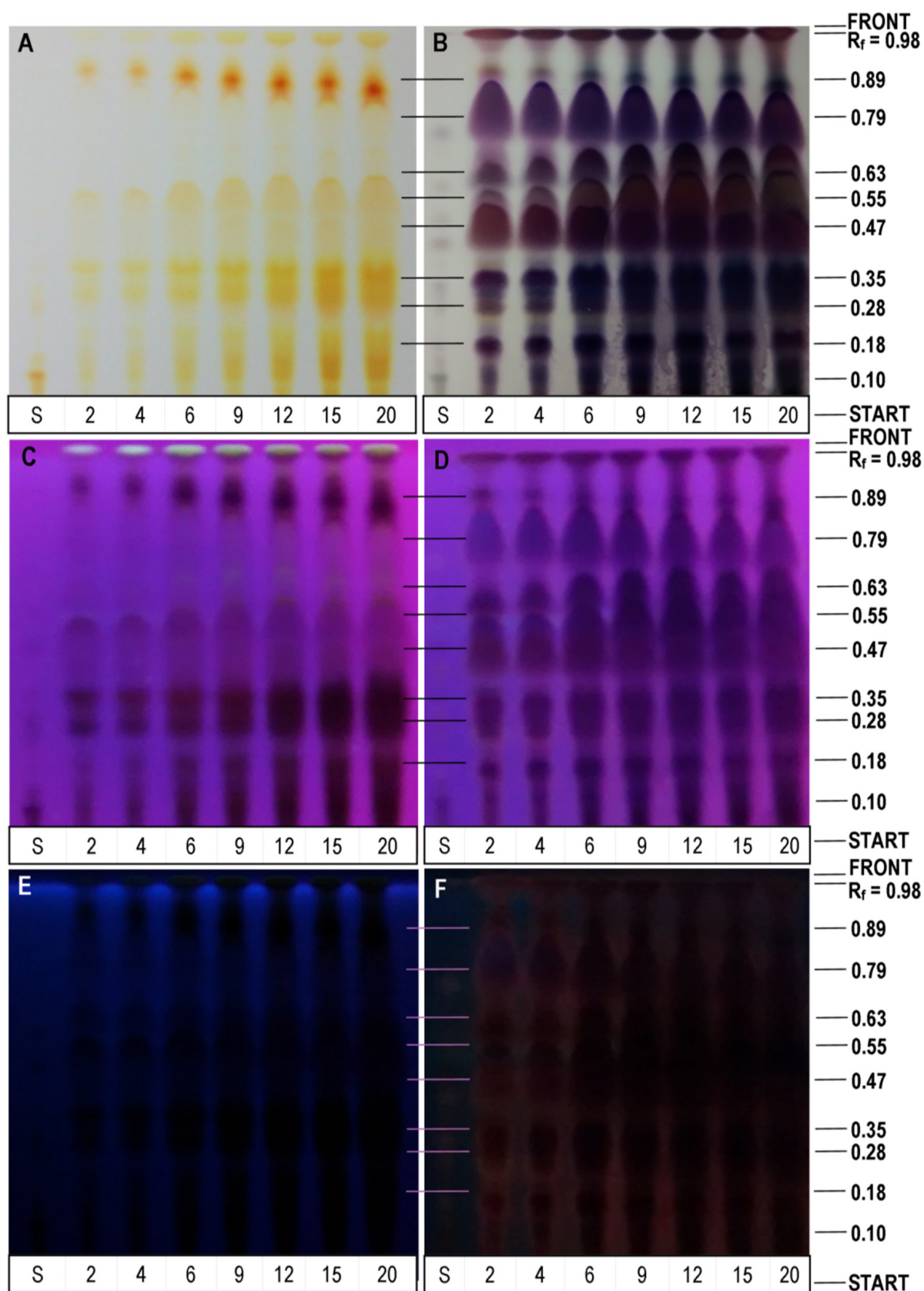
In the images of Figure 10, the  $R_f$  of 0.98 for all of the annatto extracts showed some distinct pixel intensities on the plate at 366 nm and 245 nm and, with the use of the reagent AS, revealed the presence of these compounds in all of the samples in the visible range. The type of fluorescence of image C is not characteristic of either terpenoids or polyphenols (Wagner & Bladt, 2001). The plate without developer presented an increased  $R_f$  concentration with increasing extraction time, which may indicate that there are different compounds eluted in this same  $R_f$  characteristic because they have less affinity with the mobile phase hexane:ethyl acetate:formic acid (79:18:3).

For the  $R_f$  0.89 and 0.55 (Figure 10A), the concentration of the compounds increased with extraction time; the same behavior was observed at  $R_f$  of 0.10 and 0.35 for the plate in Figure 10B.

The use of the AS developer made it possible to visualize the various compounds that could not be observed in Figure 10A, which appear in Figure 10B at  $R_f$  values of 0.18, 0.28, 0.47, 0.63 and 0.79. The AS reagent is a developer of volatile oils that doubles



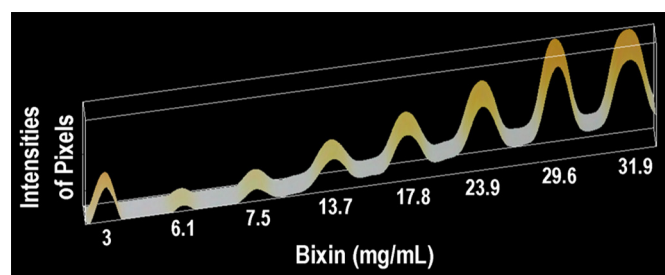
**Figure 9.** Validation steps of the bixin determination method. (A) Image of plate chromatography refers to method repeatability; (B) Image A manipulated with ImageJ (Interactive 3D Surface Plot - Thermal LUT); (C) Image of the chromatographic plate refers to method linearity; (D) Image C manipulated with ImageJ (Plot Lanes); (E) Graphic of bixin concentrations by areas of the pixels obtained by ImageJ; (F) Image C manipulated with ImageJ (Interactive 3D Surface Plot - Spectrum LUT).



**Figure 10.** Image of the chromatographic plates obtained from the elutions of the extracts from the kinetic points of Figure 8 (Lower codes: S - Bixin Standard and the respective numbers in minutes on the kinetic points). Photo A (Without Developer - Visible), photo B (With Developer - Visible), photo C (Without Developer - 366 nm), photo D (With Developer - 366 nm), photo E (Without Developer - 254 nm) and photo F (With Developer - 254 nm).



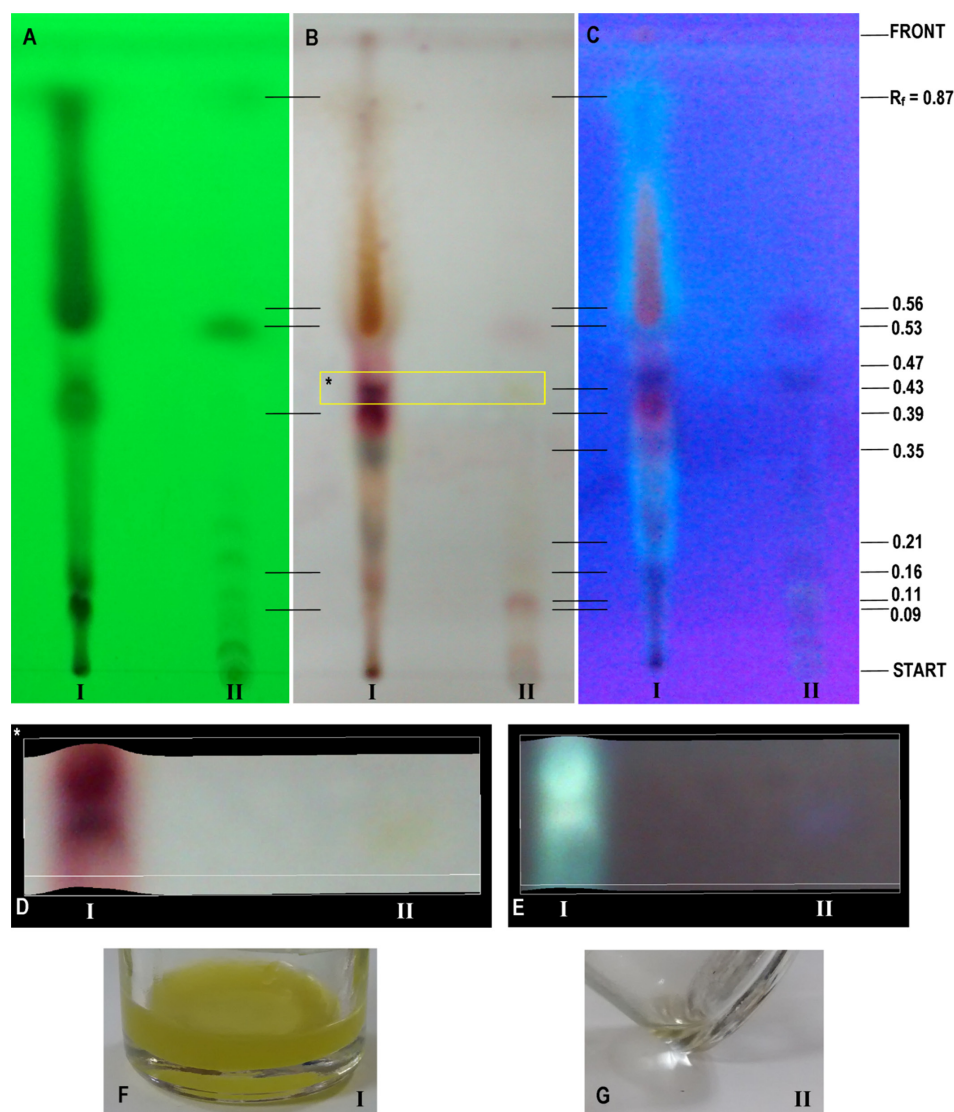
the quantity of compounds in the visible range for the annatto extracts, with the blue, green, red and brown colorations being characteristic of the essential oils (Wagner & Bladt, 2001); this confirms the presence of geranilgeraniol in annatto extracts.



**Figure 11.** Image selected from Figure 10A ( $R_f = 0.1$ ) manipulated with ImageJ (Interactive 3D Surface Plot - Original Colors).

The fennel extracts collected (Figure 12) in Separator 1 (I) and Separator 2 (II) have different compositions. The chromatography plate shows the different compositions of the extracts retained in each separator. The blade (\*) plotted on the 3D surface reinforces the differences in extract compositions, where (\*)  $R_f = 0.43$  corresponds to the fenchone present in the extract obtained in Separator II.

The method of Wagner & Bladt (2001) for developing volatile oils using concentrated sulfuric acid found that the fenchone to be sprayed should be revealed by a yellowish coloration at  $R_f \sim 0.5$ , if it is present at concentrations higher than 100  $\mu\text{g}$ . After the chromatographic plate was sprayed with this reagent, the plate presented a coloration close to that of fenchone, which is difficult to visualize at low concentrations. However, by using the Invert tool in ImageJ, which inverts image colors, it is possible to more clearly identify the compound.



**Figure 12.** Images of the chromatographic plates and of the extracts obtained from the fennel seeds from separators I and II. Photo A (Without developer - 254 nm), photo B (With developer - Visible), photo C (With Developer - 366 nm), photo D (Image B\* manipulated ImageJ - Interactive 3D Surface Plot - Original Colors), photo E (Image D manipulated with ImageJ - Invert), photo F (Extracted from the first separator) and photo G (Extract from the second separator).



Of the 2.8% yield of fennel extract, 97.5% was a waxy extract collected in the first separator, and 2.5% was an oily extract collected in the second separator. As observed in Figure 12 in the images of extracts I and II, these extracts presented distinct visual characteristics, with these being the first indicators showing the effectiveness of the separators. The work of Coşge et al. (2008) analyzed the oil of sweet fennel and found 3% essential oil, with anethole, estragole and fenchone being the predominant components of this fraction, and found 93% of monounsaturated fatty acids in the oleoresin fraction.

The extract collected in the first separator is formed by an oleoresin that also contains red-violet anisaldehyde,  $R_f = 0.39$ , and anethole,  $R_f = 0.87$ . The extract from the second separator is formed exclusively by essential oils and presented fenchone,  $R_f = 0.43$  (Figure 12E), and two other compounds,  $R_f = 0.53$  (Figure 12A) and  $R_f = 0.11$  (Figure 12B).

## 4 Conclusions

The assembled equipment may be used as a standard for a low-cost SFE unit for the formulation of projects to construct new extraction units. The extractor is adequate for use in obtaining extracts of diverse raw materials, and its separators can function to obtain fractionated extracts. The validated method for bixin quantification and the procedure adapted for the production of a bixin pattern represent a low-cost option for annatto extract analysis. The description of the use of the free ImageJ software may be adapted for analysis of other images that need precise measurements, whether they are intended to calculate  $R_f$  or not, such as the use of the tools to calculate areas from parts of images.

## Acknowledgements

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**Appendix A.** SFE-0.1L Unit Standard Operating Procedures (SOPs). Campinas-SP, July of 2015.

## Standard Operating Procedure

Knowledge of all the components of the unit is an important step in operating the equipment. SFE-0.1L unit consists of the following:

- 1 structure formed by aluminum profile 30 × 30 mm (Sistema, Amparo, Brazil)
- 2 swivel casters without brakes connected to the structure (Colson, 3"s, Araucária, Brazil)
- 2 swivel casters with brakes connected to the structure (Colson, 3"ç, Araucária, Brazil)
- 1 aluminum plate (Autic, Campinas, Brazil) that serves as the foundation for the heating and cooling baths (770 × 570 × 2 mm)
- 1 stainless steel plate that serves as the foundation for the valve box with heating system (Maq'nagua, Serra Negra, Brazil)
- 1 valve panel formed by a stainless steel plate with a size of 320 × 270 × 2 mm (Maq'nagua, Serra Negra, Brazil)
- 1 heating valve box (310 × 260 × 120 mm) for the valve panel with fluid inlet and outlet with diameter of 22 mm (Maq'nagua, Serra Negra, Brazil)
- 1 backpressure valve (Tescon, 26-1700, Sorocaba, Brazil)
- 1 backpressure valve (Tescon, 44-2200, Sorocaba, Brazil)
- 1 backpressure valve (Tescon, 44-1800, Sorocaba, Brazil)
- 1 air pressure regulator (Norcren, R07-100-RNKA, São Paulo, Brazil)
- 1 safety valve (Swagelok, SS-4R3AS, São Paulo, Brazil)
- Tube adapter, tube OD 1/8" e NPT female 1/4" (Swagelok, SS-400-6-2, São Paulo, Brazil)
- 1 air-driven CO<sub>2</sub> pump (Maximator, M-111L, Nordhausen, Germany)
- Tube 1/8" OD of 0.89 mm length (Fopil, A.I.ASTM A269TP316S, Campinas, Brazil)
- Tube adapter - Union Tee Connector, tube OD 1/8" with ferrule (Fopil, ASTM A276TP316, Campinas, Brazil)
- Tube adapter - Straight Union, tube OD 1/8" with ferrule ASTM A276TP316 (Fopil, A.I.ASTM A269TP316S, Campinas, Brazil)
- Tube adapter - tube OD 1/8" e NPT Male 1/4" ASTM A276TP316 (Fopil, A.I.ASTM A269TP316S, Campinas, Brazil)
- Tube adapter - union tee male branch NPT 1/4" (Fopil, ASTM A276TP316, Campinas, Brazil)
- Cylinder outlet connection (Hoke 7115F4Y, Spartamburg, EUA)
- Blocking valve (Autoclave Engineers, 10V2071, PA, USA)
- Micrometering valve (Autoclave Engineers, 10VRMM2812, PA, USA)
- Particulate filter (Swagelok, série F, São Paulo, Brazil)
- 2 pressure gauge - 100 bar (WIKA, EN8371-1, Klingenberg, Germany)
- 2 pressure gauge - 600 bar (WIKA, EN8371-1, Klingenberg, Germany)
- 1 pressure gauge - 400 bar (WIKA, EN8371-1, Klingenberg, Germany)
- Insulation sheets - 13 mm (Epex, Vidoflex M2, Blumenau, Brazil)
- 1 temperature indicator 173.2 K — 473.0 K (Pyrotecautomação, T4WM-TP100, Campinas, Brazil)
- 3 thermocouples - 50 mm × 1.2 m (Pyrotecautomação, PT 100, Campinas, Brazil)
- 2 thermocouples - 150 mm × 1.2 m (Pyrotecautomação, PT 100, Campinas, Brazil)
- 1 separator with heating jacket, 90 mL
- 1 separator with cooling jacket, 90 mL
- 1 extractor with heating jacket, 100 mL
- 1 ramp clamp (Keck SK, Max. tubing o.d. 1/4 in. / 6 mm, Munich, Germany)

- 2 ball valve (Japi, globo, Jundiaí, Brazil)
- Flowmeter (Cole Parmer, PMR1, IL, USA)
- Flow totalizer (Itrón, G25, Americana, Brazil)
- Silicone hose (Sinergia, 200 × 200, Campinas, Brazil)
- Insulation tube 1/8" (Armaflex, AFM-10, Campinas, Brazil)
- Insulation tube Ø - 22 mm (Isolan, Campinas, Brazil)
- 2 serpentine tubes 1/8"
- 1 hose serpentine
- 1 steel plate (420 × 350 × 2 mm)
- 2 hose npt adapter (female 22 mm) 1/2" (Amanco, Campinas, Brazil)
- 2 Hose Tee connector 1/2" (Amanco, Campinas, Brazil)
- 1 extractor support structure (Autic, Campinas, Brazil)
- 4 separators support structures (Autic, Campinas, Brazil)
- 1 aluminum plate 110 x 270 x 5 mm (Autic, Campinas, Brazil)
- 1 heating bath (1500 W) 243 K — 373 K (Thermo Haake, C10, Eindhoven, Holland)
- 1 cooling bath (2000 W) 278 K — 368 K (Thermo Haake, DC30/DL30, Eindhoven, Holland)

Each topic presented in the SOP is numbered according to the sequence of the unit operational steps and may be followed by the underlined word "Caution", with an explanation of what should be checked.

### Standard procedure to turn on and operate the SFE-0.1L unit

- 1) Turn on the air conditioning of the extraction room for approximately 10 minutes before starting work on the unit to operate at constant room temperature; record this value;
- 2) Record the value of the flow totalizer (Figure 1A);

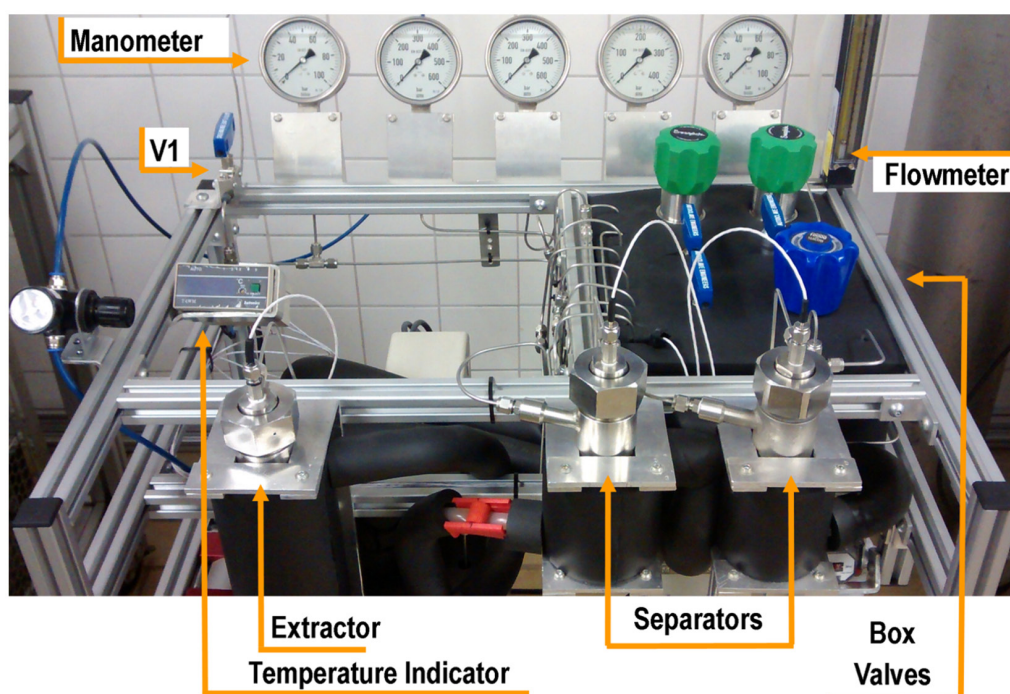
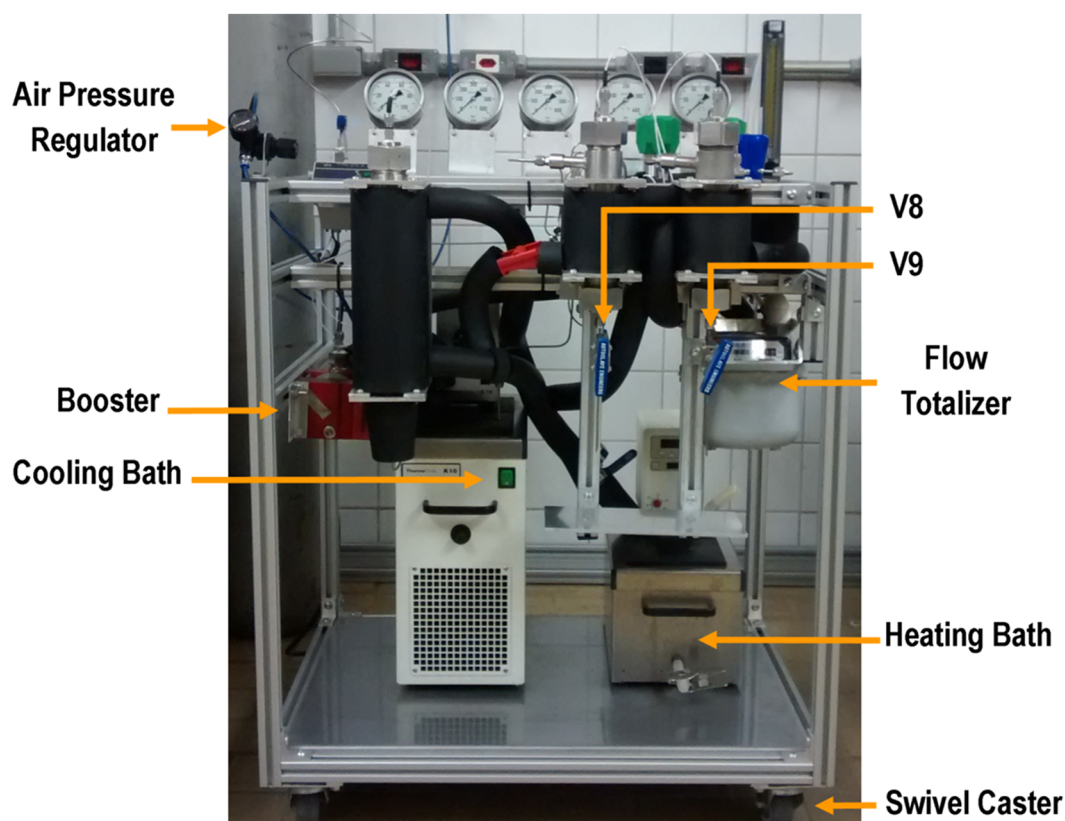


Figure 1A. Unit SFE-0.1L, front view.



- 3) Connect the power cables of the temperature indicator, thermostatic bath and heating bath in voltage and amperage indicated in the plugs (Figures 1A and 2A);
- 4) Record the temperature of the temperature indicator (Figure 2A);
- 5) Release the pressure of the compressed air to the regulator (Figure 1A);
- 6) Close the blocking valve V1 (Figure 2A);
- 7) Open the CO<sub>2</sub> cylinder valve and the ball valve of the cylinder connector that carries the CO<sub>2</sub> to the tubes (Figure 3A);
- 8) Close the blocking valve V3 (Figure 4A);
- 9) Turn on the thermostatic bath until it reaches operating temperature. The maximum working temperature must be 268 K and the minimum 255 K. The thermostatic bath takes approximately 45-60 minutes to reach the temperature;
- 10) Disconnect the 1/8" tube of the hose t connector connected to the upper part of the extractor with a 55 mm wrench with a movement of 90° counterclockwise; after this rotation no longer use the wrench, but finish with the hands (Figure 5A). Caution: If is not possible to finish opening using the hands, the thread has been damaged. Send the cover and the thread for repair before it is no longer possible to open it using a 55 mm wrench. To open the unit cell, it must be under internal pressure conditions similar to the environment to prevent damage to their threads. The covers are made of the same material as the threads, and they could be caught in the thread if torque were applied with a key while the cell was pressurized. At the slightest sign of trouble while opening, send the parts for repair;
- 11) Place the desired raw material inside the extractor;
- 12) Place the Teflon gasket into the cell and then screw the cover with your hands until the point where is necessary to use the wrench. Place the center output of the hose's T connector that connects the cover to the tubing and the thermocouple with a 45 ° angle relative to the tube that connects the 1/8 tube (Figure 5A). Exert torque on the cover clockwise with a 55 mm wrench until the connection aligns with the 1/8" tubing and screw the tubing into the hose's T connector;
- 13) Open the blocking valve V1; the cylinder manometer pressure should reach 60 bar (Figure 6A), in case the cylinder is full. (Only use the cylinder with pressure between 60 and 50 bar);



**Figure 2A.** Unit SFE-0.1L, perspective view.

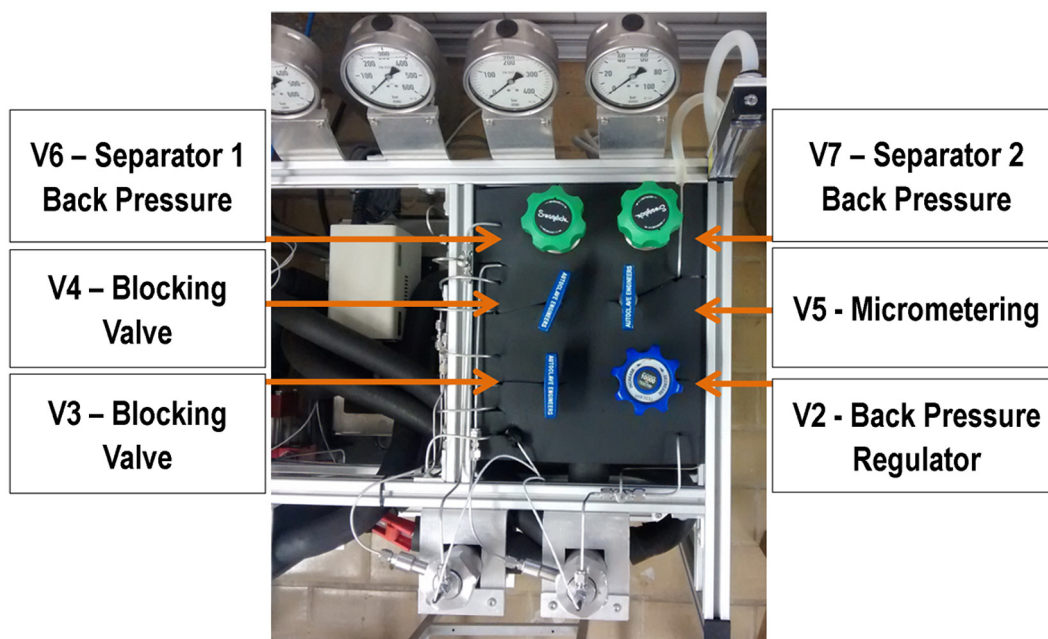


Figure 3A. CO<sub>2</sub> ball valve.

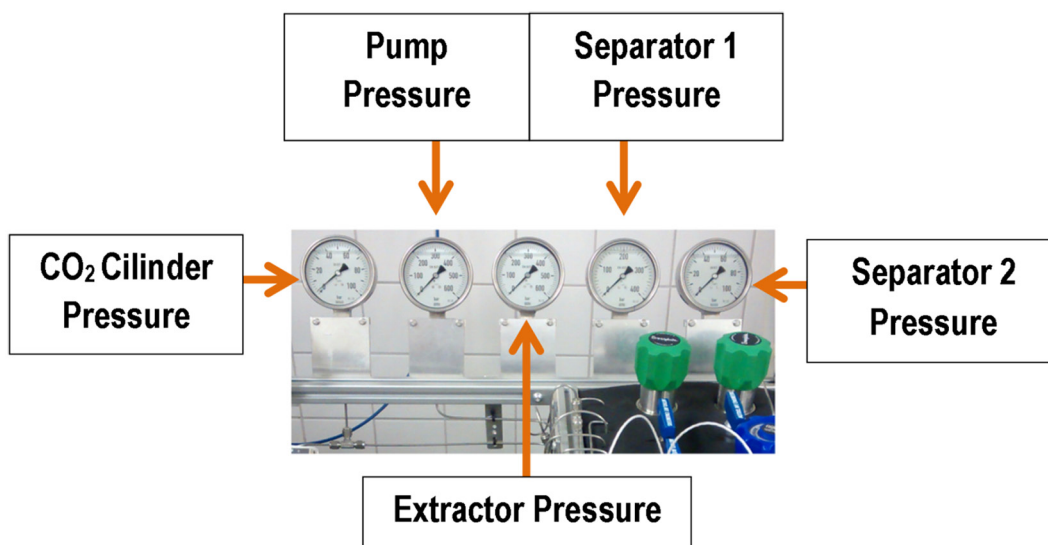
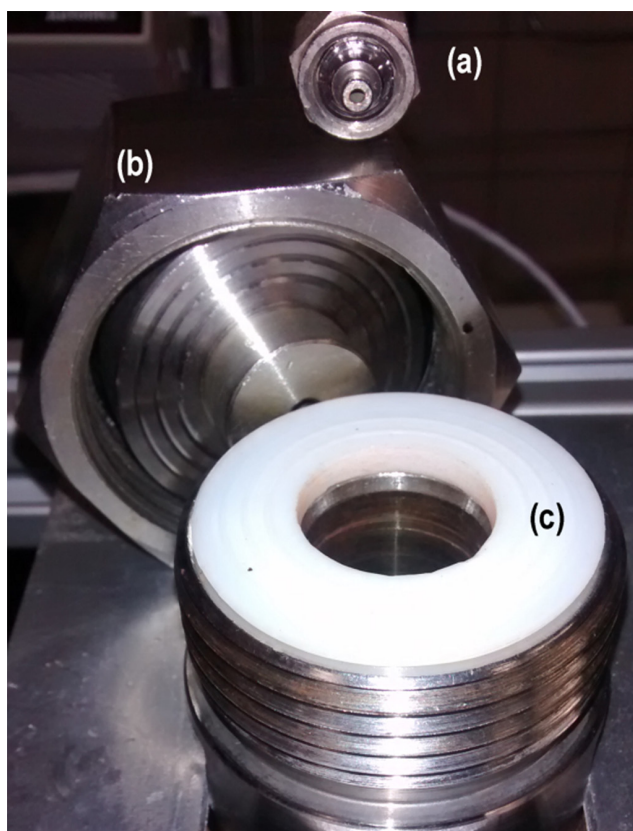


Figure 4A. Box valves.

- 14) Before turning on the heating bath (Figure 1A), the water level should be checked. Additionally, observe if the water is clean (Figure 7A); because the jacket is made of copper, the fluid should be periodically changed to avoid rusting of the thermocouple, which could affect the temperature control. Indeed, some dirty particles could damage the pump of this component. Caution: The fluid level of the heating bath should be higher than the black plastic connected to the tube and always lower than the cover of this component (Figure 7A). If the fluid level is not between the indicated positions, the bath will turn off automatically and the fluid will return to the valve panel, causing an overflow. To avoid this inconvenience, two ball valves at the base of the bath pump output (Figure 8A) and a type of hose valve with an open body were installed;
- 15) Turn on the heating bath. (This heating bath interchanges thermal energy with the extractor jacket, with the jacket of the first separator and with the valve panel). Because of the specifications of the backpressure valves, the maximum temperature of the heating bath should be 353 K. Check that the bath temperature reaches the desired temperature in the extractor. If necessary during the extraction, regulate the bath temperature to keep the temperature of the thermocouple at the extractor outlet constant;

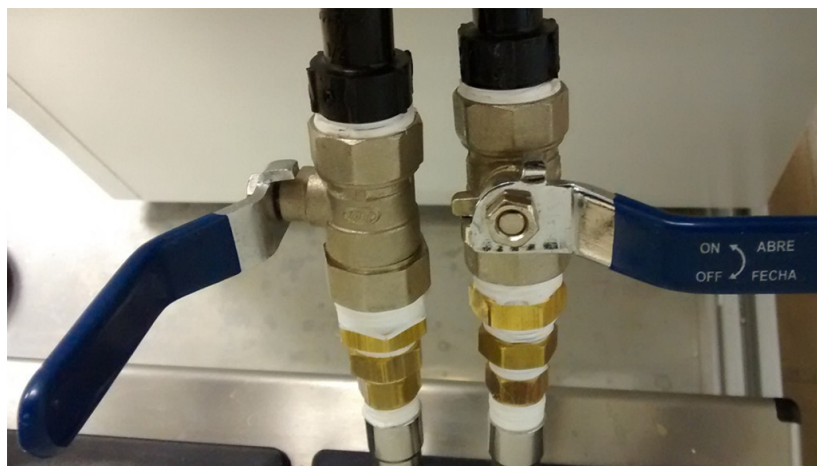


**Figure 5A.** Extractor components. (a) CO<sub>2</sub> inlet 1/8 tube; (b) Extractor - 55 mm; and (c) Teflon.

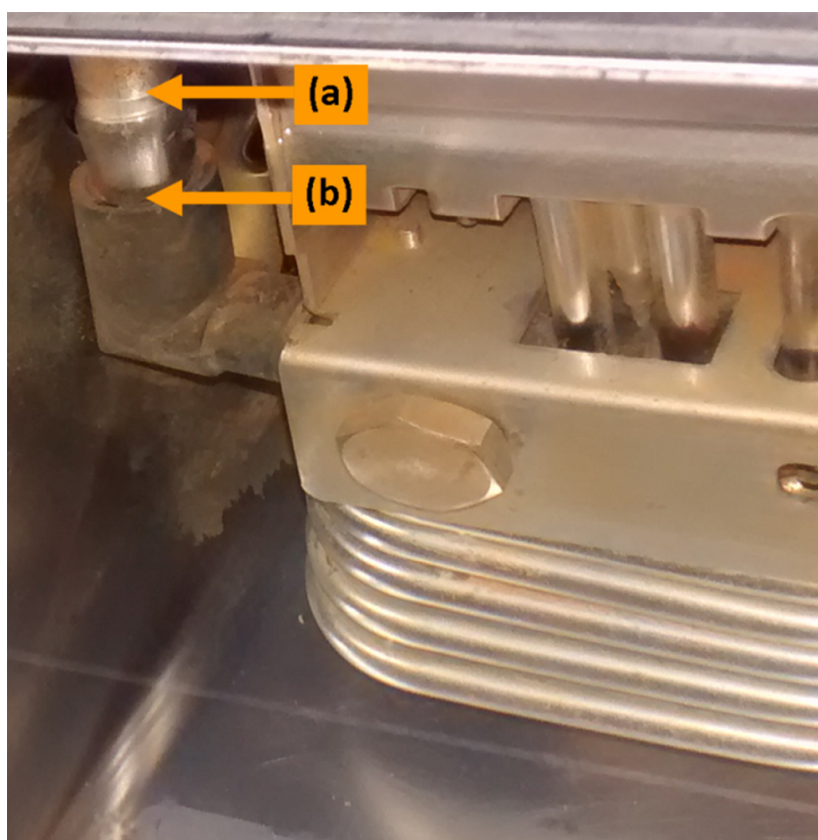


**Figure 6A.** Manometers.





**Figure 7A.** Bath level. (a) Full mark, do not overfill; (b) Add mark, add water when level is at or below this mark.



**Figure 8A.** Ball valve for water control.

16) Then, pressurize the tube that connects the air-driven CO<sub>2</sub> pump to the valve V3. Slowly release the air pressure to the air-driven CO<sub>2</sub> pump, which should begin to move its piston at 2.5 bar of pressure with the pump regulator gauge (Figure 1A). Because the backpressure valve is still fully open, the pressure should not rise in the second manometer and the flow corresponding to the excess pressure should be recirculated to the tubing before the air-driven CO<sub>2</sub>. At this point, the backpressure valve should start to slowly close; the pressure in the second manometer will rise. The air-driven CO<sub>2</sub> pump should begin to pressurize (pump) slowly and then more pressure should be liberated from the compressed air regulator until the desired pressure is reached and the CO<sub>2</sub> is recycled by the backpressure valve. (Caution: the air-driven CO<sub>2</sub> pump should work constantly to avoid pressure changes in the compressed air that cause changes in the extractor pressure. Thus, after the initial adjustment, the equipment should work constantly until the end of the extraction process without the need for controlling the pressure of the compressed air or the back pressure, Figure 4A);



- 17) Once the pump has been working constantly for some seconds, recycling the flow corresponding to the excess pressure after the pump, the blocking valve V4 should be closed (Figure 4A). Then, slowly open the blocking valve (Figure 4A) until the pressures in the extraction and pump manometers are the same. Caution: Do not open the blocking valve V3 suddenly; if this happens, the pressure after the air-driven CO<sub>2</sub> pump will fall sharply and it will pump unevenly, which may cause undesired operation;
- 18) Count 20 minutes of static time;
- 19) The micrometering valve V5 must be closed carefully. Do not close it totally and damage its needle. Caution: The micrometering valve should never be completely closed; for this reason, a blocking valve is installed in the same line for the sole purpose of performing this function. In this way, when the blocking valve is opened, the micrometering valve V5 is already in the working position and only requires an initial adjustment to maintain the flow rate;
- 20) After the static time and with the micrometering valve already adjusted to control the flow (this point corresponds to approximately 45° of rotation before the flow is blocking; never completely close the valve to avoid damaging the needle), release the blocking valve V3;
- 21) Record the beginning of the extraction time;
- 22) At this time, the flow adjustment should be performed only through the micrometering valve to keep it constant during the entire process. Caution: Considering that the fluid should pass across two separators before entering the flowmeter, a few seconds are spent before observing some change in the flowmeter caused by a position change in the micrometering valve. To avoid damage to the needle, it is necessary to wait for the response in the flowmeter before adjusting the valve V5 again (Figure 4A). Then, wait for approximately 15 seconds to evaluate if the flow in the flowmeter is constant;
- 23) Once the flow rate has been regulated, begin to close the backpressure valve V6; this valve should be closed by turning it clockwise 45° until the desired pressure in the manometer is reached (Figure 6A). Caution: This sort of valve has better precision when it is closing than when it is opening; thus, do not pressurize the system beyond the working pressure;
- 24) Then repeat the procedure described in 23) with the backpressure valve V7, which should be regulated with less pressure than the first separator. Never use excessive torque on any valve, especially on the backpressure valves; their springs are very sensitive and approximately 1200 ° rotation is enough to reach pressures of 200 bar;
- 25) Following the specified procedures, the unit should be operated without the necessity for any adjustment. If there is some variation in the flowmeter, repeat 22) to 24) while the extracts are being stored in the separators.

#### **SFE-0.1L unit shutdown procedure:**

- 1) Once the extraction time is finished, close the blocking valve V4 (Figure 4A);
- 2) Close the compressed air feeding valve (Figure 1A);
- 3) Close the blocking valve V3 (Figure 4A);
- 4) Close the CO<sub>2</sub> cylinder valve (Figure 4A);
- 5) Turn off the thermostatic bath (Figure 1A);
- 6) Record the flow totalizer value (Figure 1A);
- 7) The pressure should fall in the extractor when the air-driven CO<sub>2</sub> pump has stopped. Then carefully open the valves V4 and V5, trying to maintain a constant flow rate, to depressurize the system. Once the extractor pressure is the same as that in the first separator, open the micrometering valve V5 totally;
- 8) Gradually open the backpressure valve V6, maintaining the flowmeter at the same process flow rate until the pressures equalize in both separators;
- 9) Gradually open the back pressure valve V7, maintaining the flowmeter at the same process flow rate until 5 bar is reached in the three cells, extractor, separator 1 and 2;
- 10) Recover the extracts using proper flasks, previously weighed;
- 11) Adjust the collector base extract based on the flask height;
- 12) Open the valves V8 and V9; the remaining pressure will facilitate the recovery of the extract (Figure 1A).
- 13) Record the flow totalizer value corresponding to the end of the depressurization process;

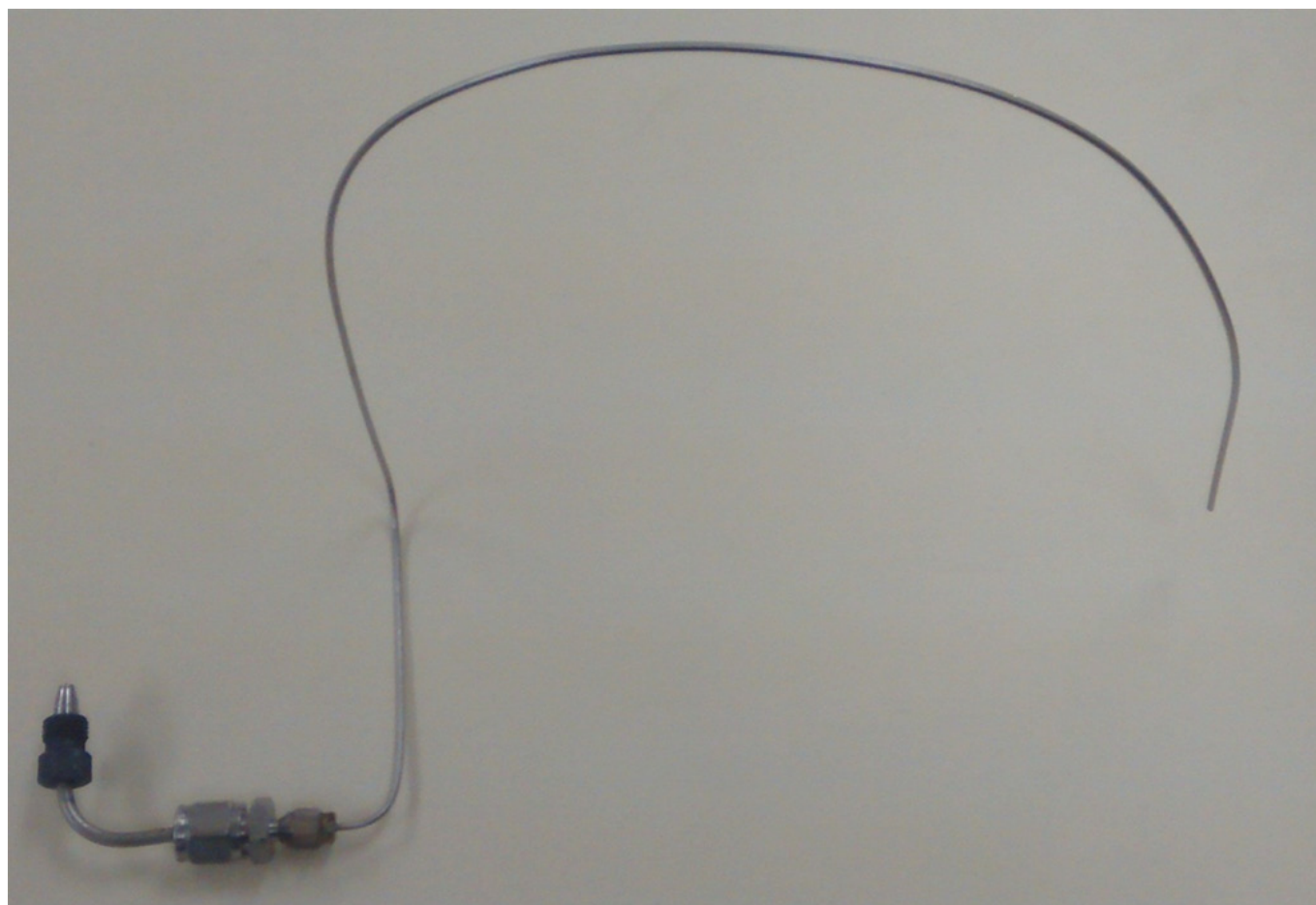
- 14) Open the 1/8" tubing that connects with the hose T connector. The pressure will fall to the ambient pressure. With the extractor still warm, open the top cover and remove the raw material;
- 15) Turn off the heating bath and immediately close both blocking valves at the pump outlet (Figure 8A) to avoid heating fluid reflux;
- 16) Perform the cleaning procedure described in the SFE-0.1L Unit Standard Cleaning Procedure;
- 17) Disconnect the power cables of the temperature indicator, thermostatic bath and heating bath.

**Appendix B.** SFE-0.1L Standard Operating Procedures (SOPs) of the Secondary Extraction Line. Campinas-SP, July of 2015

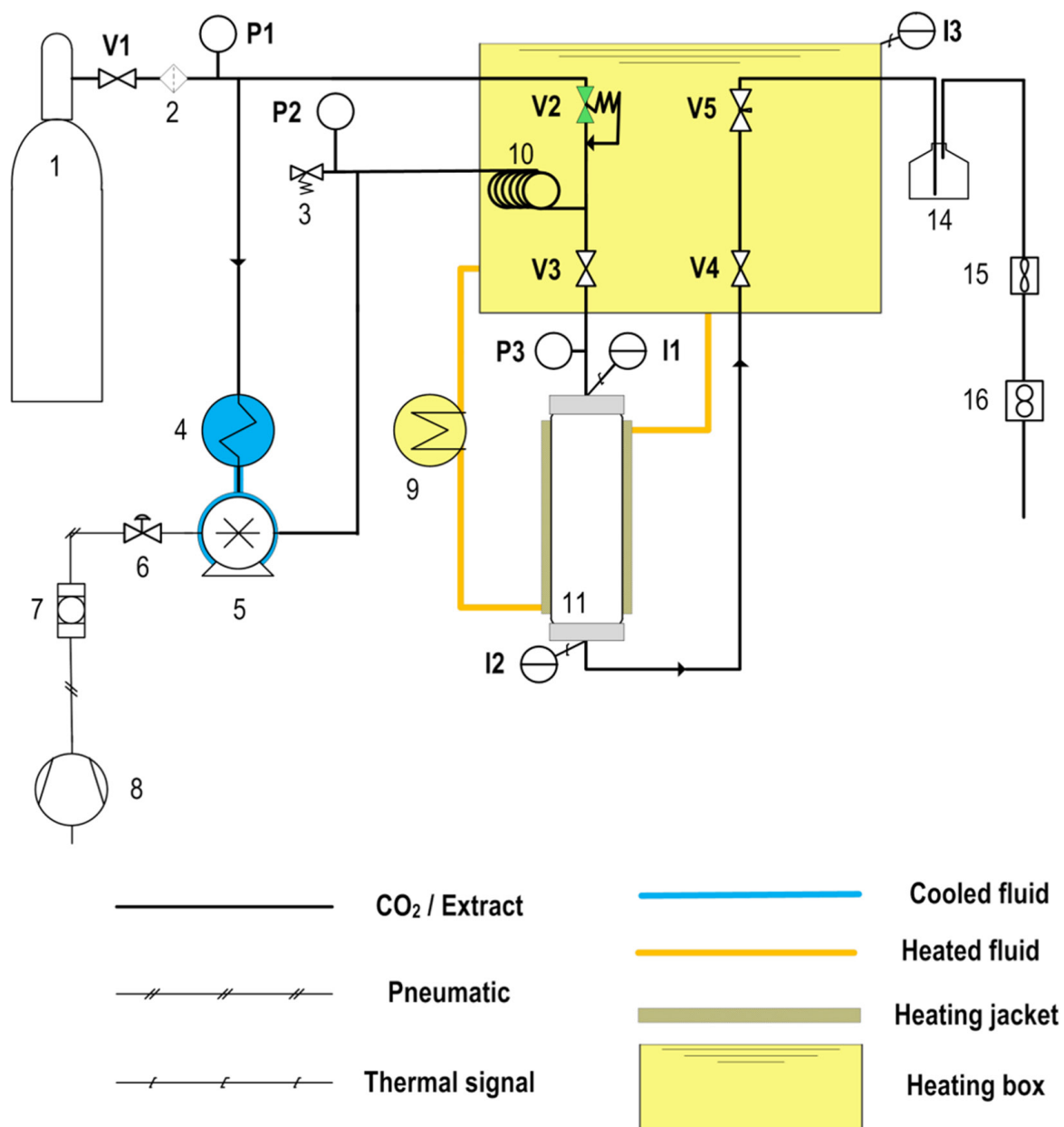
**Standard Operating Procedures (SOPs) for the secondary extraction line**

To perform studies in the SFE-0.1L unit without using the separators or if the objective is to build the unit without installing the separators, a secondary extraction line was developed. This line consists of a 1/8" tube connected to the outlet of the micrometering valve that should be inserted into a collecting flask connected after the flowmeter and flow totalizer.

Figure 1B shows the tube used in the procedure, with an angle to allow the tube to exit the valve panel; the extract is thus recovered. The details of the secondary extraction line are shown in Figure 2B; all equipment components are arranged in a flow diagram.



**Figure 1B.** Line adapter for secondary extraction.



List of equipments				List of valves	
1	CO <sub>2</sub> reservoir	11	Extractor	V(1,3,4)	Blocking valve
4	Cooling bath	14	Extrac collecting vessel	V5	Micrometering valve
5	Air-driven CO <sub>2</sub> pump	<b>List of instruments</b>			
7	Air filter	15	Flowmeter	V2	Back pressure (600 bar)
8	Air compressor	16	Flow totalizer	3	Safety valve
9	Heating bath	I(1,2,3) - Temperature Indicator			
10	Serpentine tube	P(1,2,3) - Pressure gauge			
6	Control (air flow)				

Figure 2B. Diagram of the secondary extraction line.

## Standard Operating Procedure

The knowledge of all unit components is a key factor in proper operation of the equipment. The secondary extraction line consists of the following:

- 1 structure formed by an aluminum profile 30 × 30 mm (Sistema, Amparo, Brazil)
- 2 swivel casters without brakes connected to the structure (Colson, 3" s, Araucária, Brazil)
- 2 swivel casters with brakes connected to the structure (Colson, 3" c, Araucária, Brazil)
- 1 aluminum plate (Autic, Campinas, Brazil) that serves as the foundation for the heating and cooling baths (770 × 570 × 2 mm);
- 1 stainless steel plate that serves as the foundation for the valve box with heating system (Maq'nagua, Serra Negra, Brazil)
- 1 valve panel formed by a stainless steel plate of size of 320 × 270 × 2 mm (Maq'nagua, Serra Negra, Brazil)
- 1 heating valve box (310 × 260 × 120 mm) for the valve panel with fluid inlet and outlet with diameter of 22 mm (Maq'nagua, Serra Negra, Brazil)
- 1 backpressure valve (Tescon, 26-1700, Sorocaba, Brazil)
- 1 backpressure valve (Tescon, 44-2200, Sorocaba, Brazil)
- 1 backpressure valve (Tescon, 44-1800, Sorocaba, Brazil)
- 1 air pressure regulator (Norcren, R07-100-RNKA, São Paulo, Brazil)
- 1 safety valve (Swagelok, SS-4R3AS, São Paulo, Brazil)
- Tube adapter, tube OD 1/8" e NPT female 1/4" (Swagelok, SS-400-6-2, São Paulo, Brazil)
- 1 air-driven CO<sub>2</sub> pump (Maximator, M-111L, Nordhausen, Germany)
- Tube 1/8" OD 0.89 mm (Fopil, A.I.ASTMA269TP316S, Campinas, Brazil)
- Tube adapter - Union Tee Connector, tube OD 1/8" with ferrule (Fopil, ASTM276TP316, Campinas, Brazil)
- Tube adapter - Straight Union, tube OD 1/8" with ferrule ASTM A276TP316 (Fopil, A.I.ASTMA269TP316S, Campinas, Brazil)
- Tube adapter - tube OD 1/8" e NPT Male 1/4" ASTM A276TP316 (Fopil, A.I.ASTMA269TP316S, Campinas, Brazil)
- Tube adapter - union tee male branch NPT 1/4" (Fopil, ASTM276TP316, Campinas, Brazil)
- Cylinder outlet connection (Hoke 7115F4Y, Spartamburg, EUA)
- Blocking valve (Autoclave Engineers, 10V2071, PA, USA)
- Micrometering valve (Autoclave Engineers, 10VRMM2812, PA, USA)
- Particulate filter (Swagelok, série F, São Paulo, Brazil)
- 2 pressure gauges - 100 bar (WIKA, EN8371-1, Klingenberg, Germany)
- 2 pressure gauges - 600 bar (WIKA, EN8371-1, Klingenberg, Germany)
- 1 pressure gauge - 400 bar (WIKA, EN8371-1, Klingenberg, Germany)
- Insulation sheets - 13 mm (Epex, Vidoflex M2, Blumenau, Brazil)
- 1 temperature indicator 173.2 K — 473.0 K (Pyrotecautomação, T4WM-TP100, Campinas, Brazil)
- 3 thermocouples - 50 mm × 1.2 m (Pyrotecautomação, PT 100, Campinas, Brazil)
- 2 thermocouples - 150 mm × 1.2 m (Pyrotecautomação, PT 100, Campinas, Brazil)
- 1 separator with heating jacket, 90 mL;
- 1 separator with cooling jacket, 90 mL
- 1 extractor with heating jacket, 100 mL;
- 1 ramp clamp (Keck SK, Max. tubing o.d. 1/4 in. / 6 mm, Munich, Germany)
- 2 ball valve (Japi, globo, Jundiaí, Brazil)



- Flowmeter (Cole Parmer, PMR1, IL, USA)
- Flow totalizer (Itrón, G25, Americana, Brazil)
- Silicone hose (Sinergia, 200 × 200, Campinas, Brazil)
- Insulation tube 1/8" (Armaflex, AFM-10, Campinas, Brazil)
- Insulation tube Ø - 22 mm (Isolan, Campinas, Brazil)
- 2 serpentine tube 1/8";
- 1 serpentine hose;
- 1 steel plate (420 × 350 × 2 mm);
- 2 hose npt adapters (female 22 mm) 1/2" (Amanco, Campinas, Brazil)
- 2 Hose Tee connectors 1/2" (Amanco, Campinas, Brazil)
- 1 extractor support structure (Autic, Campinas, Brazil)
- 4 separators support structures (Autic, Campinas, Brazil)
- 1 aluminum plate 110 x 270 x 5 mm (Autic, Campinas, Brazil)
- 1 heating bath (1500 W) 243 K — 373 K (Thermo Haake, C10, Eindhoven, Holland)
- 1 cooling bath (2000 W) 273 K — 368 K (Thermo Haake, DC30/DL30, Eindhoven, Holland)

Each topic presented in the SOP is numbered according to the sequence of the unit operational steps, and may be followed by the underlined word "Caution" with the explanation of what should be checked.

#### **Standard procedure to turn on and operate the SFE-0.1L unit with the secondary extraction line:**

- 26) Turn on the air conditioning of the extraction room for approximately 10 minutes before starting work on the unit to operate at constant room temperature; record this value;
- 27) Record the value of the flow totalizer (Figure 3B);
- 28) Connect the power cables of the temperature indicator, thermostatic bath and heating bath in voltage and amperage indicated in the plugs (Figures 3B and 4B);
- 29) Record the temperature of the temperature indicator (Figure 4B);
- 30) Release the pressure of the compressed air to the regulator (Figure 3B);
- 31) Close the blocking valve V1 (Figure 4B);
- 32) Open the CO<sub>2</sub> cylinder valve and the ball valve of the cylinder connector that carries the CO<sub>2</sub> to the tubes (Figure 5B);
- 33) Close the blocking valve V3 (Figure 6B);
- 34) Turn on the thermostatic bath until it reaches operating temperature. The maximum working temperature must be 268 K and the minimum 255 K. The thermostatic bath takes approximately 45-60 minutes to reach the temperature;
- 35) Disconnect the 1/8" tube of the hose t connector connected to the upper part of the extractor with a 55 mm wrench with a movement of 90° counterclockwise; after this rotation no longer use the wrench but finish with the hands (Figure 7B). Caution: If is not possible to finish opening using the hands the thread was damaged; send the cover and the thread for repair before it is no longer possible to open it using a 55 mm wrench. To open the unit cell, it must be in internal pressure conditions similar to the environment to prevent damage to their threads. The covers are made of the same material as the threads, and they could be caught into the thread if torque were applied with a key while the cell is pressurized. At the slightest sign of trouble during the opening, send the parts for repair;
- 36) Place the desired raw material inside the extractor;
- 37) Place the Teflon gasket into the cell and then screw the cover with your hands until the point where it is necessary to use the wrench. Place the center output of the hose tee connector that connects the cover to the tubing and the thermocouple with a 45 ° angle relative to the tube that connects the 1/8" tube (Figure 7B). Exert torque on the cover clockwise with a 55 mm wrench until the connection aligns with the 1/8" tubing and screw the tubing into the hose's t connector;

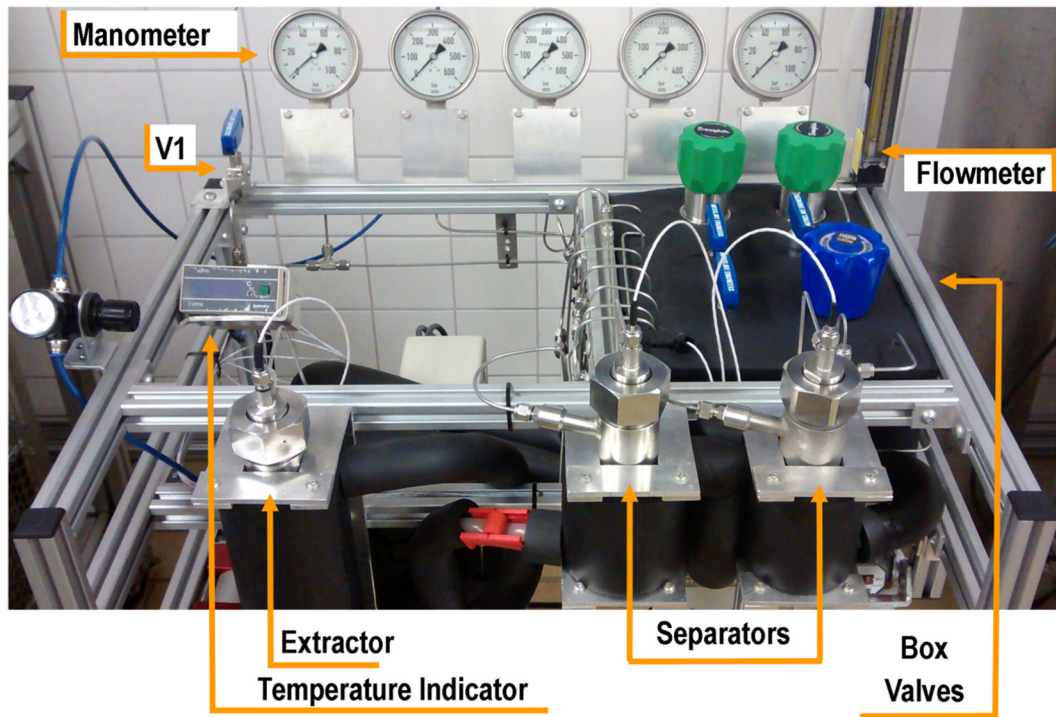


Figure 3B. Unit SFE-0.1L, front view.

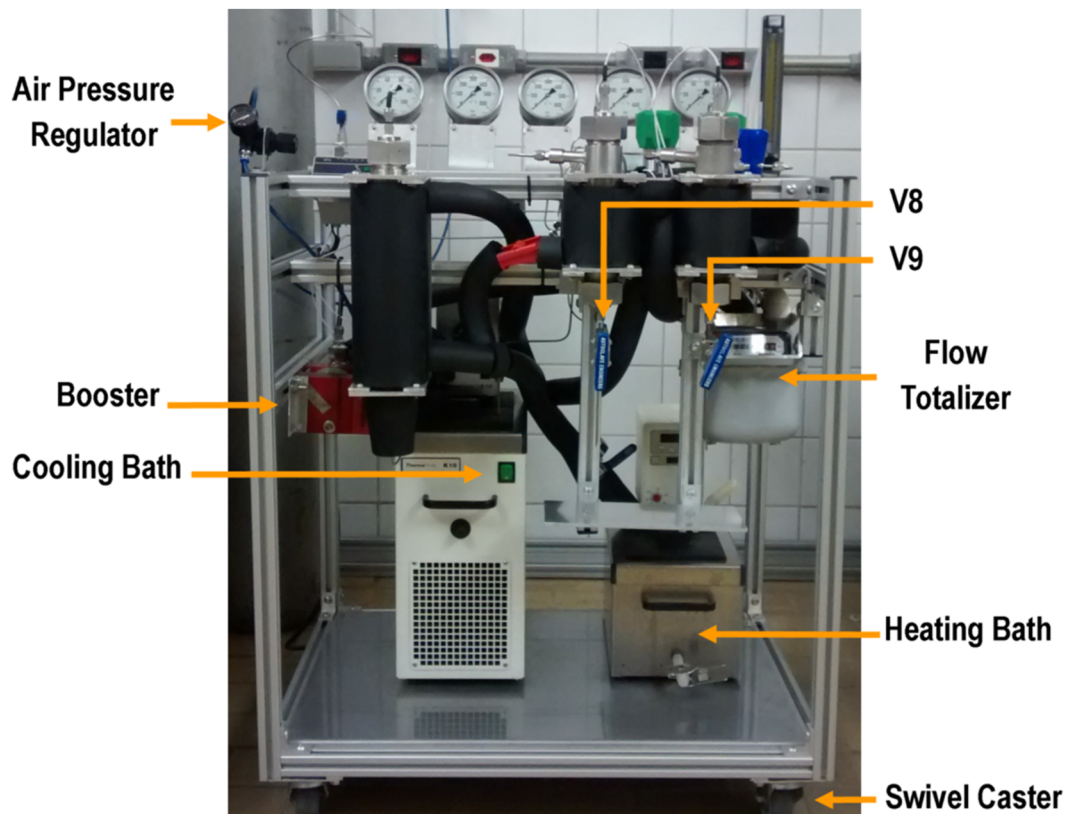


Figure 4B. Unit SFE-0.1L, perspective view.

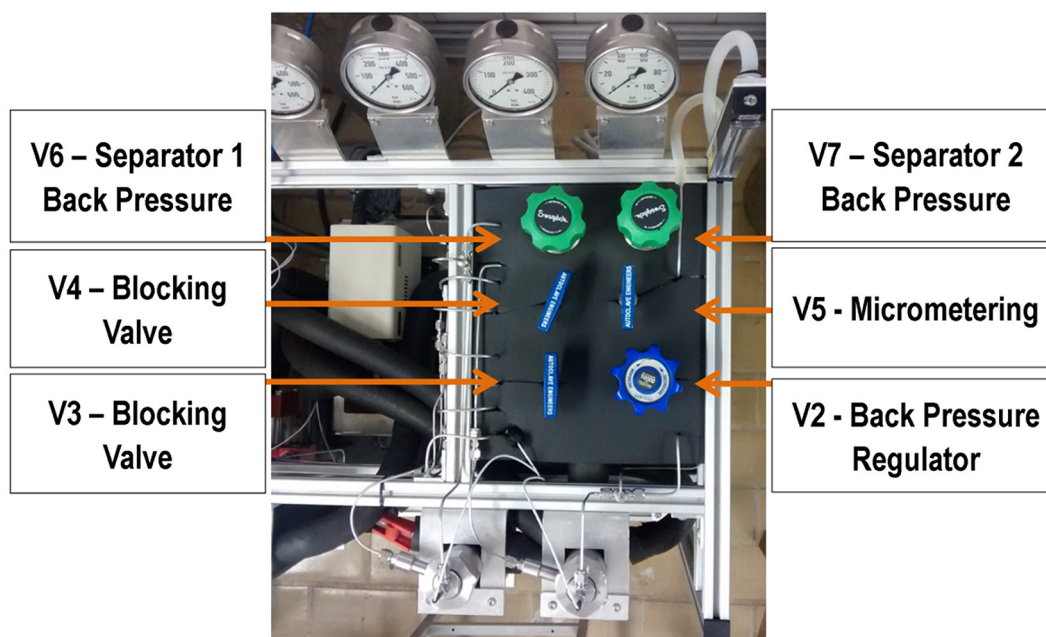


Figure 5B. CO<sub>2</sub> ball valve.

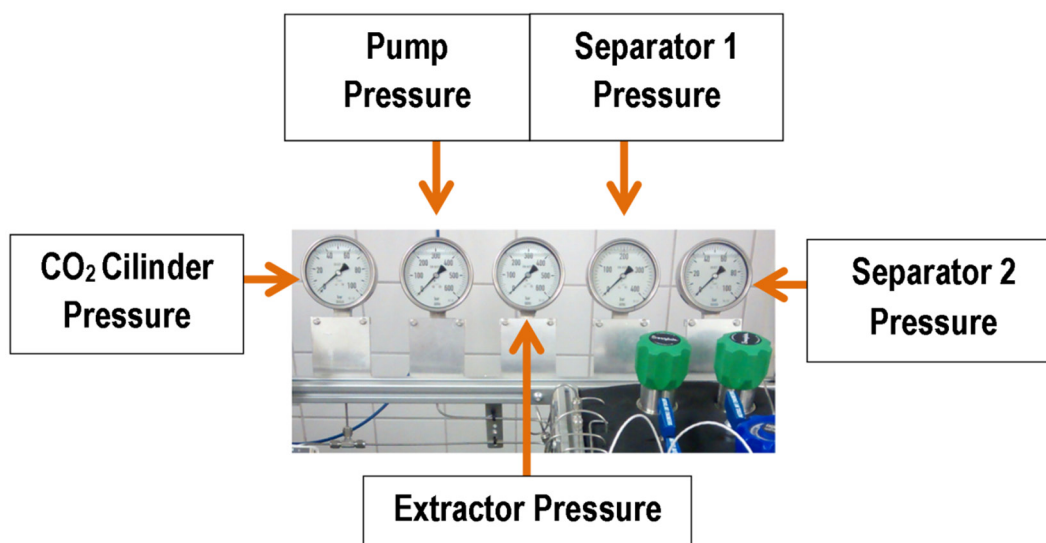
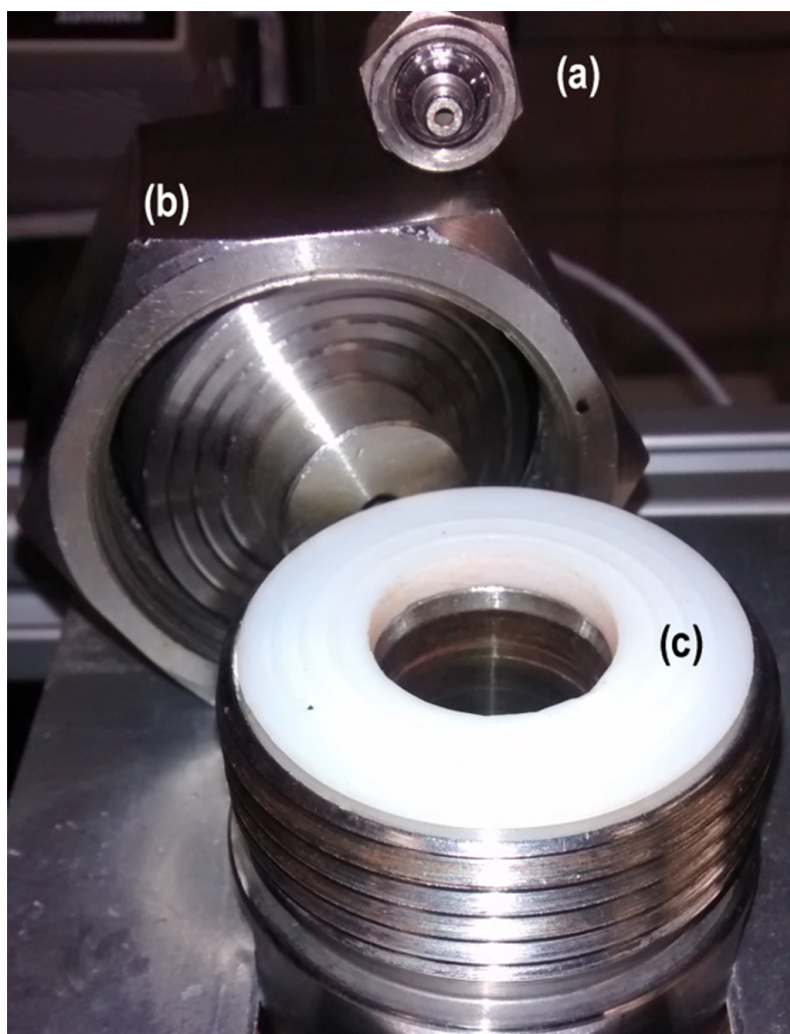


Figure 6B. Box valves.

- 38) Open the blocking valve V1; the cylinder manometer pressure should reach 60 bar (Figure 8B), in case the cylinder is full (only use the cylinder with pressure between 60 and 50 bar);
- 39) Before turning on the heating bath (Figure 3B), the water level should be checked. Additionally, observe if the water is clean (Figure 9B); because the jacket is made of copper, the fluid should be periodically changed to avoid rusting of the thermocouple, which could affect the temperature control. Indeed, some dirty particles could damage the pump of this component. Caution: The fluid level of the heating bath should be higher than the black plastic connected to the tube and should always be lower than the cover of this component (Figure 9B). If the fluid level is not between the indicated positions, the bath will turn off automatically and the fluid will return to the valve panel, causing overflow. To avoid this inconvenience, two ball valves at the base of the bath pump output (Figure 10B) and a type of hose valve with an open body were installed;





**Figure 7B.** Extractor components, (a) CO<sub>2</sub> inlet 1/8 tube; (b) Extractor - 55 mm; and (c) Teflon.

- 40) Turn on the heating bath. (This heating bath interchanges thermal energy with the extractor jacket and with the valve panel). Because of the specifications of the backpressure valves, the maximum temperature of the heating bath should be 353 K. Check that the bath temperature reaches the desired temperature in the extractor. If necessary during the extraction, regulate the bath temperature to keep the temperature of the thermocouple at the extractor outlet constant;
- 41) Then, pressurize the tube that connects the air-driven CO<sub>2</sub> pump to the valve V3. Slowly release the air pressure to the air-driven CO<sub>2</sub> pump, which should begin to move its piston at 2.5 bar of pressure with the pump regulator gauge (Figure 3B). Because the backpressure valve is still fully open, the pressure should not rise in the second manometer and the flow corresponding to the excess pressure should be recirculated to the tubing before the air-driven CO<sub>2</sub>. At this point, the backpressure valve should start to slowly close, and the pressure in the second manometer will rise. The air-driven CO<sub>2</sub> pump should begin to pressurize (pump) slowly and then more pressure should be liberated from the compressed air regulator until the desired pressure is reached and the CO<sub>2</sub> is recycled by the backpressure valve. (Caution: the air-driven CO<sub>2</sub> pump should work constantly to avoid pressure changes in the compressed air that cause changes in the extractor pressure. Thus, after the initial adjustment, the equipment should work constantly until the end of the extraction process without the need for controlling the pressure of the compressed air or the back pressure, Figure 6B);
- 42) Once the pump has been working constantly for some seconds, recycling the flow corresponding to the excess pressure after the pump, the blocking valve V4 should be closed (Figure 6B). Then, slowly open the blocking valve (Figure 6B) until the pressures in the extraction and pump manometers are the same. Caution: Do not open the blocking valve V3 suddenly; if this happens, the pressure after the air-driven CO<sub>2</sub> pump will fall sharply, and it will pump unevenly, which may cause undesired operation;
- 43) Count 20 minutes of static time;





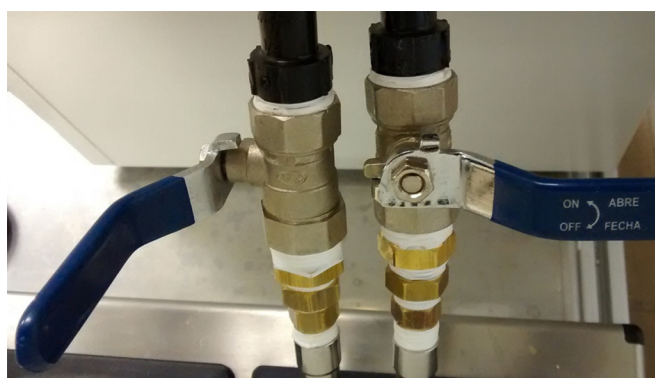
**Figure 8B.** Manometers.

- 44) The micrometering valve V5 must be closed carefully. Do not close it totally and damage its needle. Caution: The micrometering valve should never be completely closed; for this reason, a blocking valve is installed in the same line for the sole purpose of performing this function. In this way, when the blocking valve is opened, the micrometering valve V5 is already in the working position and only requires an initial adjustment to maintain the flow rate;
- 45) After the static time and with the micrometering valve already adjusted to control the flow (this point corresponds around 45° of rotation before the flow is blocking; never completely close the valve to avoid damaging the needle), release the blocking valve V3;
- 46) Record the beginning of the extraction time;
- 47) At this time, the flow adjustment should be performed only through the micrometering valve to keep it constant during the entire process. Caution: A few seconds are spent before observing some change in the flowmeter caused by a position change in the micrometering valve. To avoid damage to the needle, it is necessary to wait for the response in the flowmeter before again adjusting the valve V5 (Figure 6B). Then, wait for approximately 15 seconds to evaluate if the flow in the flowmeter is constant.

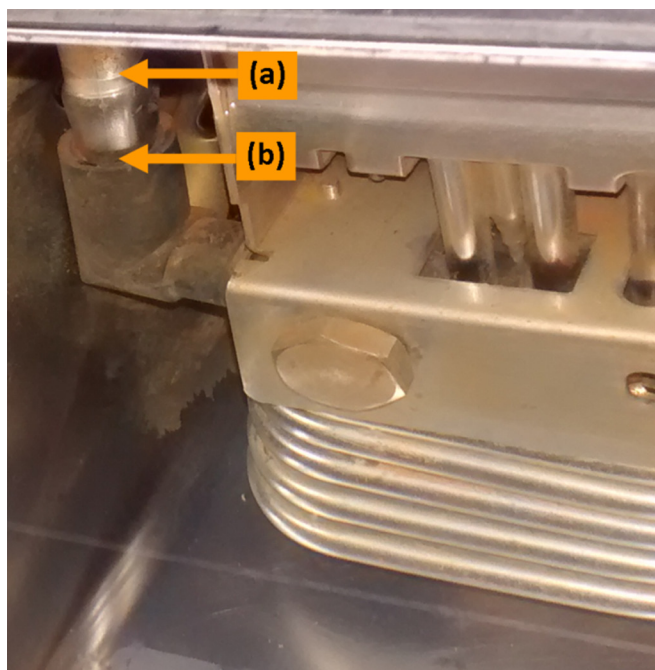
#### **SFE-0.1L unit with secondary extraction line shutdown procedure:**

- 18) Once the extraction time is finished, close the blocking valve V4 (Figure 6B);
- 19) Close the compressed air feeding (Figure 3B);

- 20) Close the blocking valve V3 (Figure 6B);
- 21) Close the CO<sub>2</sub> cylinder valve (Figure 5B);
- 22) Turn off the thermostatic bath (Figure 3B);
- 23) Record the flow totalizer value (Figure 3B);
- 24) The pressure should fall in the extractor when the air-driven CO<sub>2</sub> pump is stopped. Then carefully open the valves V4 and V5, trying to maintain a constant flow rate to depressurize the system;
- 25) Record the flow totalizer value corresponding to the end of the depressurization process;
- 26) Open the 1/8" tubing that connects to the hose t connector. The pressure will fall to the ambient pressure. With the extractor still warm, open the top cover and remove the raw material;
- 27) Turn off the heating bath and immediately close both blocking valves at the pump outlet (Figure 10B) to avoid heating fluid reflux;
- 28) Perform the cleaning procedure describe in the SFE-0.1L Unit Standard Cleaning Procedure;
- 29) Disconnect the power cables of the temperature indicator, thermostatic bath and heating bath.



**Figure 9B.** Bath level. (a) Full mark, do not overfill (b) Add mark, add water when level is at or below this mark.



**Figure 10B.** Ball valve for water control.

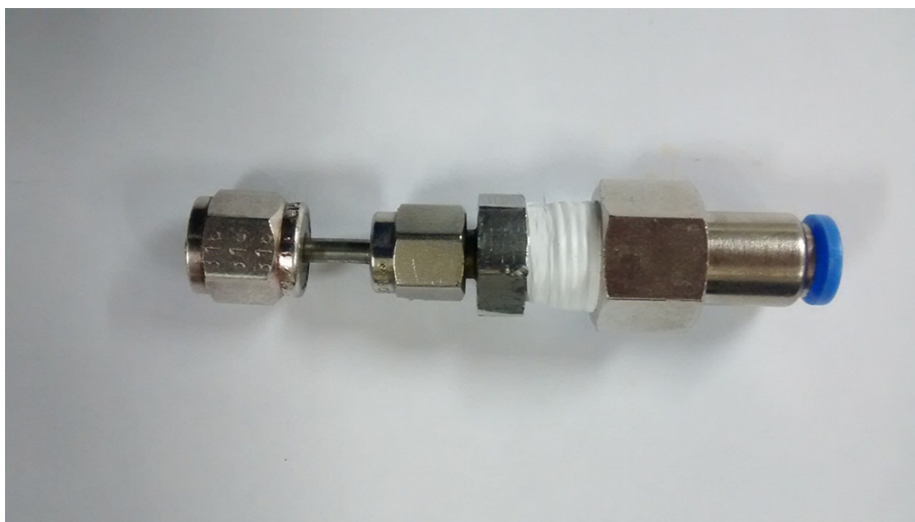
**Appendix C. SFE-0.1L Unit Standard Cleaning Procedure (SCP). Campinas-SP, July of 2015****SFE-0.1L Unit Standard Cleaning Procedure**

A connection that uses the pressure of compressed air (6-12 bar) to clean the system was developed. This connection consists of a 1/8" tube 0.04 m in length. In that tube, one end has a connector OD-OD, and the other end has a straight connector OD-NPT that is threaded to a pneumatic connection entrance with a polyurethane tube.

**Unit Standard Cleaning Procedure**

- 1) Open the extractor cover and disconnect the 1/8" tube with a wrench and then with a 55 mm wrench, turning it counterclockwise;
- 2) Remove the raw material;
- 3) Open the bottom cover with the same wrench as in step 1) but turning it clockwise;
- 4) Clean the extractor covers and the Teflon gaskets;
- 5) Close the extractor cover and connect the 1/8" tube to the extractor outlet;
- 6) Fill the extractor with a solvent that can dissolve the extract; it can be ethanol or some other solvent, depending on the extracted raw material;
- 7) Turn the cleaning connection (Figure 1C) at the upper extractor cover;
- 8) Put a collector flask at the end of the extraction line;
- 9) Connect the compressed air tube to the cleaning connection;
- 10) Pressurize the extractor with compressed air to force the flow of solvent. During the procedure is recommended that valves are open and closed for further cleaning;
- 11) Repeat this process until all extraction residues from the tubes and valves are totally removed;
- 12) After the removal of all cleaning solvent, the compressed air line should be still working for around two minutes to guarantee the removal of the remaining solvent;
- 13) Close the compressed air line;
- 14) Disconnect the cleaning connection and connect the 1/8" tube.

This procedure should be performed immediately after extraction has been performed. Thorough cleaning of pipes and valves of the equipment through this procedure ensures that no fouling is formed in the pipes and valves, avoiding clogging and reducing experimental variations.



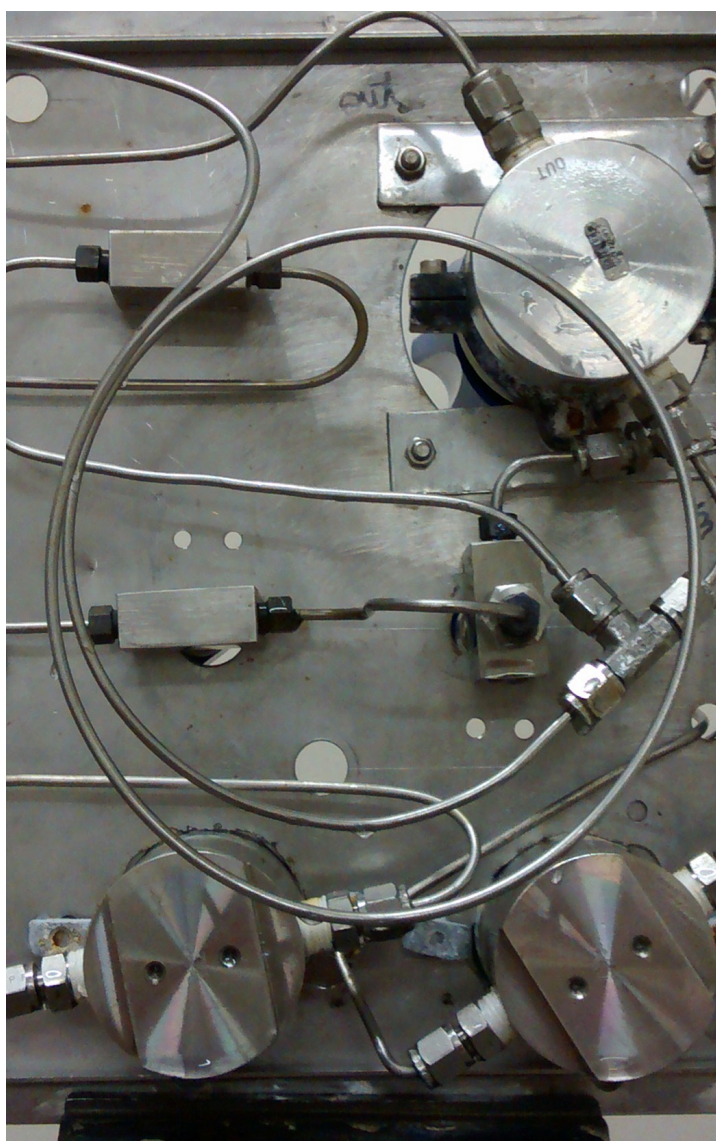
**Figure 1C.** Cleaning Connection.

**Appendix D. Micrometering Valve Standard Operating Procedures (SOPs). Campinas-SP, July of 2015****Micrometering Valve Standard Operating Procedure**

The cleaning procedure for the micrometering valve of the SFE-0.1L unit should be performed whenever this component is not responding correctly when it is operated, probably because there is some clogging. The micrometering valve is responsible for controlling the flow during the process; therefore, the lack of periodic maintenance could cause considerable variations in the experimental results.

**Micrometering Valve Standard Cleaning Procedure:**

1. Remove the valve panel to provide access to the micrometering valve;
2. Fix the connections with a monkey wrench and disconnect unions and crosses with a wrench (1/2") to release the tubes that enter into the valve panel;
3. Attach the valve panel to the table with a bench vise as shown in Figure 1D to perform the procedure quickly;
4. Before removing the valve, fully open the blue lever to avoid any damage to the needle during the procedure;



**Figure 1D.** Panel valves.



5. With an Allen wrench, remove the blue lever and the two position indicators;
6. Unscrew the valve support with a screwdriver and remove the valve near the lower part of the panel, as shown in Figure 2D;



**Figure 2D.** Micrometering valve.

7. Position the valve body with a monkey wrench or use the bench vise;
8. With a 5/8" wrench, unscrew the upper part from the lower part of the valve, as shown in Figure 3D, being careful not to miss any internal parts during the procedure. Caution: The micrometering pieces are of great precision and dropping it could damage its operation permanently;



**Figure 3D.** Opening the valve components.

9. Clean all the pieces, including the whole valve, with enough ethanol to remove any fragments that are trapped in the line at this point. Sample fragments or even valve fragments could damage the valve and cause leaking. Clean and dry all components before reassembling the valve;
10. Fit the components in the sequence shown in Figure 4D and squeeze with sufficient force to prevent leakage;



**Figure 4D.** Valve components.

11. Install the valve in the valve panel;
  12. Connect the inlet and outlet tubes;
  13. Install the valve panel in the unit;
- Connect all 1/8" tubes that were disconnected previously to perform the valve maintenance.